5. The countercurrent-distribution technique has also been used for studies of the action of aqueous HCl on protoporphyrin and its methyl ester.

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Carbohydrate Metabolism in Citric Acid Fermentation

4. PURIFICATION AND PROPERTIES OF ALDOLASE FROM *ASPERGILLUS NIGER**

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Meyerhof & Lohmann (1934) discovered and investigated in detail the enzyme aldolase which catalyses the reaction

Aldolase from rabbit muscle was purified by Herbert, Gordon, Subrahmanyam & Green (1940). The enzyme from rat muscle was later crystallized by Warburg & Christian (1943), and from rabbit muscle by Taylor, Green & Cori (1948). Warburg & Christian (1943) partially purified yeast aldolase and discovered that it required heavy metals for its activity. The enzyme was found to be inhibited by metal-binding agents, such as cysteine, pyrophosphate and *o*-phenanthroline, and reactivated by Zn^{2+} , Fe^{2+} or Co^{2+} . Muscle aldolase did not require or contain these metals and was not inhibited by any of the metal-binding agents. Subsequent studies of the enzyme with preparations of

low purity from different tissues have shown the existence of both types of aldolase. The activation of aldolase from *Escherichia coli* by Mn^{2+} (Knox, Stumpf, Green & Auerbach, 1948) and the requirement of *Clostridium perfringens* aldolase for Fe^{2+} or Co^{2+} (Bard & Gunsalus, 1950) have been reported. Partially purified pea aldolase was, however, unaffected by heavy metals or metal-binding agents (Stumpf, 1948).

During a study of the glycolytic enzymes of *Aspergillus niger* it was observed that the aldolase in crude extracts of the mould mycelium was completely inhibited by the metal-chelating agent ethylenediaminetetraacetic acid, and was reactivated by Zn^{2+} (Jagannathan & Singh, 1954). The aldolases of other moulds which were investigated (*Aspergillus oryzae*, *Aspergillus wentii*) also resembled yeast aldolase and differed from muscle aldolase in their metal requirement. Since the metal-requiring aldolases had been studied mostly in crude form, the isolation of the enzyme from

* Part 3: Damodaran, Jagannathan & Singh (1956).

A. niger in the pure state and the study of its properties were undertaken. Yeast aldolase has recently been crystallized as the mercury salt (Warburg & Gawehn, 1954).

The present paper deals with the purification and properties of *A. niger* aldolase. The purified enzyme was electrophoretically homogeneous and its specific activity was equal to that of crystalline muscle or yeast aldolase. It was inhibited by metal-binding agents and was reactivated by bivalent ions of Zn, Mn, Fe or Co.

MATERIALS AND METHODS

Materials. The following substances were commercial preparations: diphosphopyridine nucleotide (DPN) 85% pure, reduced glutathione (GSH), fructose 1:6-diphosphate (FDP), glucose 6-phosphate and fructose 6-phosphate from Schwartz Laboratories, protamine sulphate from L. Light and Co., Colnbrook, Bucks, disodium ethylenediamine-tetraacetic acid (EDTA) from Bersworth Chemical Co. and aminotrihydroxymethylmethane (tris) from Sigma Chemical Co.; FDP was further purified according to Neuberg, Lustig & Rothenberg (1943). The barium salt of β -D-acetonefructose 1-phosphate was converted into free fructose 1-phosphoric acid according to Leuthardt & Testa (1951). Dihydroxyacetone phosphate was prepared from FDP, using dialysed rabbit-muscle extract (Meyerhof & Lohmann, 1934), and was free from glyceraldehyde 3-phosphate. Calcium phosphate gel (Keilin & Hartree, 1951) was aged for 6 months or longer before use. Crystalline glyceraldehyde phosphate dehydrogenase (Cori, Slein & Cori, 1948) and aldolase (Taylor *et al.* 1948) were prepared from rabbit muscle and recrystallized till they were free from α -glycerophosphate dehydrogenase and triose phosphate isomerase. Triose phosphate isomerase was purified according to Meyerhof & Beck (1944) and was free from aldolase and α -glycerophosphate dehydrogenase. Glass-distilled water was used for all experiments.

***A. niger* mycelium.** Spores of the high citric acid yielding strain of *A. niger* used in previous studies (Damodaran & Rangachari, 1951; Jagannathan & Singh, 1953) were obtained from 7-day-old cultures on potato-dextrose-agar slants. The culture medium (1 l.) contained 120 g. of glucose (Dextrosol); 2 g. each of $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 and KH_2PO_4 ; 0.5 g. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 10 mg. of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5 mg. of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$; 0.5 mg. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 8 ml. of 2N-HCl (final pH 1.8). It was sterilized by steaming for 1 hr. The dry spores were transferred by gentle tapping to 4 l. Pyrex Haffkine culture flasks, each containing 1 l. of medium and incubated in still culture at 28° for 7 days. The mycelium which was free from spores was filtered through cheese-cloth and squeezed to remove as much of the adhering liquid as possible. It was then washed four to five times with ice water till the pH of the filtrate was about 5 or higher. The mycelium was stored for 3-4 weeks at -18° before use. The average yield of mycelium from twenty flasks was about 1.6 kg. (fresh weight).

Analytical methods. Inorganic phosphate was estimated by the method of Fiske & Subbarow (1925) as modified by Lohmann & Jendrasick (1926), iron by the dipyriddy method, zinc by the dithizone method and manganese by oxidation to permanganate (Sandell, 1950). FDP was

estimated spectrophotometrically by measuring the total DPN reduced in the presence of crystalline muscle aldolase, triose phosphate dehydrogenase and triose phosphate isomerase in the test system described in the text (EDTA and Zn^{2+} omitted); 1 μmole of FDP was equivalent to 2 μmoles of reduced DPN under these conditions. Protein was determined by the optical method of Warburg & Christian (1941). The following empirical equation was used to correct for light absorption due to nucleic acid, the light path being 1 cm.:

$$\frac{1}{2}[2.3 \times (\text{optical density at } 280 \text{ m}\mu) - (\text{optical density at } 260 \text{ m}\mu)] = \text{mg. of protein/ml. of solution.}$$

This equation was found to be in good agreement with the dry weight of dialysed purified enzyme samples and with their protein content calculated from total nitrogen (micro-Kjeldahl) and total carbon (micro-combustion) on the assumption that the protein contained N, 16%, and C, 53%.

Measurement of pH was made with the glass electrode and optical measurements were carried out in a Beckman Quartz Spectrophotometer (Model DU, 1 cm. cuvettes) with an arrangement to maintain the temperature within $\pm 1^\circ$. The effect of inhibitors on aldolase under anaerobic conditions was studied in cuvettes similar to Thunberg tubes, which were alternately evacuated and filled with hydrogen thrice. Centrifugings were carried out in an International Refrigerated Centrifuge (Model PR-1) and electrophoresis experiments in a Tiselius Electrophoresis Apparatus (Model 38, Perkin Elmer Corporation) with a Longworth scanning device.

Aldolase assay. Aldolase activity was determined by the optical method of Warburg & Christian (1943). The test system was essentially the same as in their method except that Zn^{2+} and EDTA were added to it and muscle glyceraldehyde phosphate dehydrogenase was used instead of the enzyme from yeast. The solution contained 0.9 μmole of DPN, 60 μmoles of cysteine, 50 μmoles of sodium arsenate, 100 μmoles of tris, 200 $\mu\text{g.}$ of crystalline glyceraldehyde phosphate dehydrogenase, 0.54 μmole of EDTA, *A. niger* aldolase, 0.8 μmole of zinc sulphate and 50 μmoles of FDP in a final volume of 3 ml. All the reagents were at pH 7.6 and the temperature was maintained at 30°. Aldolase preparations were diluted with ice-cold 0.1M tris buffer at pH 7.6 immediately before use. The first six components were incubated for 5 min. and the remaining components were then added in the order given above. The increase in optical density (ΔE) at 340 m μ was measured at 30 sec. intervals for 4 min. after the addition of FDP. The amount of DPN reduced/min. was calculated from the mean of these readings, the extinction coefficient of reduced DPN being taken as $6.22 \times 10^4 \text{ cm}^2 \times \text{mole}^{-1}$ at 340 m μ . All experiments were carried out in duplicate. Modifications in the method of assay are indicated in the text. A new spectrophotometric method for aldolase estimation is described below.

A unit of aldolase activity was defined as the amount of enzyme which catalysed the reduction of 1 μmole of DPN/min. at 30° under the above conditions. In the absence of triose phosphate isomerase the reduction of 1 μmole of DPN was equivalent to the conversion of 1 μmole of FDP into 1 μmole each of the two triose phosphates. The specific activity of the enzyme was defined as the activity/mg. of enzyme.

Ammonium sulphate fractionation. The quantity of $(\text{NH}_4)_2\text{SO}_4$ required for changing the degree of saturation

was calculated from the following equation modified from that of Kunitz (1952):

$$X = 50(S_2 - S_1)/(1 - 0.28S_2),$$

where X equals g. of solid $(\text{NH}_4)_2\text{SO}_4$ to be added to 100 ml. of solution of saturation S_1 in order to change it to saturation S_2 , S_1 and S_2 being expressed in fractions of saturation at 0° . The equation for changing the degree of saturation of a solution by adding saturated $(\text{NH}_4)_2\text{SO}_4$ soln. was

$$Y = 100(S_2 - S_1)/(1 - S_2),$$

where Y equals ml. of saturated solution to be added to 100 ml. of a solution to change its saturation from S_1 to S_2 . Analytical grade $(\text{NH}_4)_2\text{SO}_4$ was used except during the first precipitation (fraction B , see below), for which Merck's 'laboratory reagent' grade was used. A saturated solution of the salt was prepared by dissolving 70.6 g. in 1 l. of water. Solutions saturated at pH 7.2 and 7.6 were obtained by adding 0.60 and 1.35 ml. respectively of 2N- NH_3 soln. to 100 ml. each of the saturated solution, no correction being made for the resulting dilution. (The pH values of these solutions were measured directly without dilution and do not correspond with the actual values.) The degree of saturation of a solution obtained by dissolving a protein precipitate in a definite volume of water was calculated from the volume of the precipitate and the salt concentration at which it was precipitated as described in the text. Ammonium sulphate, solid or solution, was added slowly with stirring and the liquid was allowed to stand for 30–60 min. before centrifuging. The salt saturation and the values of pH etc. have been used for obtaining rapid and reproducible results, and do not necessarily correspond exactly with the theoretical values.

RESULTS

Isolation of aldolase

Extraction. The frozen mycelium (4.2 kg.) was rapidly broken up into small lumps, mixed with 4.2 l. of 0.01N- NH_3 soln. and allowed to stand at room temperature for 1 or 2 hr. with occasional stirring. When all the frozen mycelium had thawed (temp. about 1°), the material was squeezed through muslin. The opalescent filtrate was reddish brown and its pH was 6.3–6.5 (fraction A).

Ammonium sulphate fractionation. All further operations were carried out between 0° and 5° . The extract was brought to 0.8 saturation with $(\text{NH}_4)_2\text{SO}_4$ (51.5 g./100 ml.) and filtered with gentle suction on large Büchner funnels (Whatman no. 1 filter paper, 24 cm.). Filtration was rapid and 4 l. could be filtered within an hour on two funnels. The precipitate was suspended in 700 ml. of ice water (final volume 736 ml., 0.04 saturation) and the $(\text{NH}_4)_2\text{SO}_4$ saturation was increased to 0.50 (26.7 g./100 ml.). The precipitate was removed by centrifuging (in the angle head for 45 min. at 3000 rev./min.) and discarded. The supernatant was brought to 0.80 saturation (19.3 g./100 ml.) and centrifuged. The precipitate containing aldolase was dissolved in 140 ml. of ice water and brought to pH 7.2 with 2N- NH_3 soln. (fraction B).

Saturated $(\text{NH}_4)_2\text{SO}_4$ soln., pH 7.2, was then added to the enzyme to give a final saturation of 0.60. The precipitate was centrifuged and discarded, and the supernatant was brought to 0.80 saturation by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ [12.9 g. of $(\text{NH}_4)_2\text{SO}_4$ and 0.15 ml. of 2N- NH_3 soln./100 ml.]. The precipitate was centrifuged and stored at -18° . The entire procedure up to this stage was carried out during the same day. Fractionation between 0.60 and 0.80 saturation was then repeated as before. The initial concentration of aldolase was not greater than 80 units/ml. and the final centrifuging was carried out in the high-speed head (18 000 g for 20 min.), to remove as much of the adhering liquid as possible. The final precipitate (fraction D) could be kept at -18° for several months with no loss in activity.

Protamine treatment. Fraction D was dissolved in a small amount of water and the solution was adjusted to pH 6.0 by the addition of about 10 ml. of 0.5M acetate buffer of pH 5.6. It was then treated with sufficient protamine sulphate (1.6 g. of protamine sulphate dissolved in a solution containing 154 ml. of water and 5.6 ml. of 0.5M acetate buffer of pH 5.8) till no further precipitation occurred. In general, 1 mg. of protamine sulphate was required for 1 mg. of protein. After 30 min. the precipitate, which contained no aldolase activity, was centrifuged and discarded. The ratio of the optical density of the solution (fraction E) at 280 $m\mu$. to that at 260 $m\mu$. (280/260 ratio) increased from about 0.7 to 1.3 during this step, indicating the removal of most of the nucleic acid.

The supernatant, fraction E , from the protamine nuclein precipitate was treated with calcium phosphate gel. The concentration of $(\text{NH}_4)_2\text{SO}_4$ in the enzyme solution before the addition of the gel was adjusted to 0.02–0.05 saturation as aldolase absorption was optimum within these ranges. Increase in pH or decrease in temperature was avoided to prevent precipitation of protamine or its complexes. The calcium phosphate gel was diluted with acetate buffer at pH 6 to give a final concentration of 0.4M acetate and 16 mg. of calcium phosphate/ml. During washing and elution the gel was mixed in a Potter-Elvehjem homogenizer by hand for a few minutes to obtain a uniform suspension free from lumps. As the amounts of gel required to be added to fraction E varied slightly from batch to batch they were determined in preliminary experiments with small samples of the enzyme.

The final procedure to which each batch of enzyme was submitted was as follows: fraction E (containing 7000 units in about 200 ml.) was treated with 20.6 ml. of gel, stirred for 10 min. and centrifuged. After discarding the residue a further

Table 1. *Purification of aldolase**A. niger* (4.2 kg. fresh wt.) was used for this experiment.

Fraction	Vol. (ml.)	Activity (units/ml.)	Total activity (units)	Total protein (mg.)	Specific activity (units/mg.)
A. Extract	5600	2.5	14 000	42 000	0.3
B. $(\text{NH}_4)_2\text{SO}_4$ fraction, 0.5–0.8*	164	64	10 500	5 250	2.0
C. $(\text{NH}_4)_2\text{SO}_4$, 0.6–0.8*	120	67	8 040	1 960	4.1
D. $(\text{NH}_4)_2\text{SO}_4$, 0.6–0.8*	42	171	7 180	1 680	4.3
E. Protamine supernatant	206	34	7 000	1 360	5.1
F. 1st calcium phosphate gel supernatant	226	30	6 780	1 220	5.6
F ₁ . 2nd gel supernatant	288	2	50	920	—
G. Gel eluate	156	27	4 210	110	38.6
H. Final fraction	—	—	4 100	105	39.0

* These figures refer to the degree of saturation with $(\text{NH}_4)_2\text{SO}_4$.

62 ml. of gel was added to the supernatant. After stirring again for 10 min. and centrifuging the supernatant was discarded. The residual gel was washed twice with 100 ml. portions of a liquid having the following composition: 12 ml. of saturated $(\text{NH}_4)_2\text{SO}_4$ soln., 20 ml. of 0.5M acetate buffer (pH 5.8), and 380 ml. of water. The washings contained very little aldolase. The gel was then mixed with 160 ml. of 0.60 saturated $(\text{NH}_4)_2\text{SO}_4$, pH 7.6 (about 1 ml. of eluant for 50–60 units of adsorbed aldolase) and centrifuged after stirring for 30 min. The water-clear eluate (fraction *G*) was brought to 0.90 saturation by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ (20 g. of the salt and 0.6 ml. of 2N- NH_3 soln./100 ml. of eluate) and after 1 hr. was centrifuged (18 000 *g* for 1 hr.). The precipitate containing pure aldolase (fraction *H*) was stored as such at -18° . The 280/260 ratio increased from 1.3 to 1.8 or 1.9 after gel adsorption, indicating the absence of nucleic acids in the eluate.

The results of a typical experiment are recorded in Table 1. Reproducible results were obtained with a dozen different batches of mycelium. No further purification was obtained by repetition of gel adsorption or $(\text{NH}_4)_2\text{SO}_4$ fractionation. Triose phosphate isomerase, which was present up to fraction *F*, was completely removed during gel adsorption since it was not adsorbed by the gel. The activities and specific activities shown in Table 1 for all fractions except *G* and *H* tend to be higher than the actual values, since in the optical test both the trioses cause reduction of DPN when isomerase is present, whereas only glyceraldehyde 3-phosphate is estimated in the absence of isomerase. The final product contained negligible amounts of triose phosphate isomerase. Pure enzyme (1 mg.) caused no reduction of DPN in 5 min. when it was tested for isomerase in the usual assay system with 20 μ moles of dihydroxyacetone phosphate instead of FDP as substrate. The apparent loss of about 2000 units of activity during elution (difference between fraction *G* and the sum

of fractions *F* and *F*₁) was therefore due to the removal of isomerase and not to the loss of aldolase. The purified enzyme was also free from α -glycerophosphate dehydrogenase, glyceraldehyde phosphate dehydrogenase, phosphatase (FDP or dihydroxyacetone phosphate as substrate at pH 7.6) and diaphorase.

The specific activity of purified aldolase obtained from different batches of *A. niger* varied between 38 and 42 units/mg. of enzyme. The specific activities of the crystalline aldolases from rat muscle (Warburg & Christian, 1943), rabbit muscle (Baranowski & Niederland, 1949) and yeast (Warburg & Gawehn, 1954) were recalculated in terms of the units defined in this paper and are shown below (Table 3). The relative specific activity of the rabbit-muscle aldolase prepared by Herbert *et al.* (1940) is difficult to estimate since their method of enzyme assay was different. The specific activity of *A. niger* aldolase is equal to or slightly higher than that reported for the crystalline enzymes from muscle and yeast, though the activity of the fungal enzyme was determined with muscle triosephosphate dehydrogenase, which has been reported to give lower aldolase activities than yeast triose phosphate dehydrogenase, which was used by the other investigators (Baranowski & Niederland, 1949).

Electrophoresis. Electrophoretic studies of *A. niger* aldolase were carried out in slightly alkaline buffers (pH 7.6 and 8.6) since the enzyme was unstable at a lower pH. The purified enzyme was reprecipitated twice from a 1% solution of EDTA at 0.90 $(\text{NH}_4)_2\text{SO}_4$ saturation to remove zinc and other metals present in aldolase, which might cause turbidity at a higher pH. The enzyme precipitate was dissolved in the buffer used for electrophoresis and dialysed with stirring at 0° against three charges of the same buffer for 8 hr.

Electrophoresis was carried out in phosphate buffer at pH 7.6 and barbiturate buffer at pH 8.6. A single peak was obtained in both experiments,

Fig. 1. Electrophoresis diagram of aldolase in phosphate buffer, pH 7.6, ionic strength 0.2, after 240 min.

indicating the homogeneity of the enzyme. Fig. 1 shows the pattern obtained in phosphate buffer (ionic strength 0.2, pH 7.6, protein 0.57 %); the mobility of the enzyme was 5.43×10^{-5} cm.²/v⁻¹ × sec.⁻¹. In barbiturate buffer (ionic strength 0.1, pH 8.6, protein 0.65 %) the mobility was 6.4×10^{-5} cm.²/v⁻¹ × sec.⁻¹. The activity of the enzyme was not significantly altered during the period of electrophoresis.

Properties and kinetics

Aqueous solutions of *A. niger* aldolase (1–5 %) were water-clear and colourless. The solubility of the enzyme in (NH₄)₂SO₄ solutions at pH 7.6 was much higher than that of muscle aldolase. The latter can be precipitated almost completely at 0.52 saturation, whereas *A. niger* aldolase was soluble to the extent of 0.1 % in 0.70 saturated (NH₄)₂SO₄ soln. at the same pH and was appreciably soluble even at 0.80 saturation when it was free from nucleic acid. But the mould enzyme resembled muscle aldolase in being less soluble in 0.40–0.50 saturated (NH₄)₂SO₄ at 20° than at 0°.

The enzyme was very stable when the precipitates obtained from alkaline (NH₄)₂SO₄ solutions were stored as such at –18° and showed no loss in activity after 3 or 4 months. When dissolved in 0.40–0.60 saturated (NH₄)₂SO₄ at pH 7.6–8.0 the enzyme was stable for several days at 0° and could be heated at 50° for 5 min. without any loss in activity. Solutions in water or tris buffer, pH 7.6, were less stable; 0.2 % enzyme solution lost 40 % of its activity in 15 min. at 38° and was completely inactivated in 5 min. at 50°. The stability of the enzyme was not significantly different when it was reprecipitated from 1 % (w/v) EDTA solution with (NH₄)₂SO₄ or when it was dissolved in 1 % (w/v) EDTA, indicating that removal of the zinc present in aldolase (see below) does not affect its stability. The enzyme was also stable when dialysed for 24 hr. at 0° against dilute NH₃ soln. (0.005N), phosphate or veronal buffer (0.1M, pH 7.6–8.6).

Fig. 2. Ultraviolet absorption spectrum of aldolase.

But dialysis against buffers at pH 6.6 under the same conditions resulted in 75 % loss in activity. The inactivation could not be reversed by the addition of Zn²⁺ or boiled undialysed enzyme. Longer periods of dialysis against water or buffer (with or without 0.001M-ZnSO₄) almost completely inactivated the enzyme.

Absorption spectrum. The ultraviolet absorption spectrum of *A. niger* aldolase, shown in Fig. 2, is that of a typical protein.

Analysis for metals. Aldolase of specific activity 39 was analysed for Fe, Mn and Zn. Manganese was present in negligible amounts; 5 mg. of the enzyme contained less than 0.1 μg. of the metal, which was the limit of detection by the method used for analysis. The iron content of the enzyme was also low (0.07 μg./mg. of protein). If 1 mole of iron were to be present in 100000 g. of enzyme, the iron content of the preparation should be at least eight times higher. Zinc was present in significant amounts (1.3–1.4 μg./mg. of aldolase). It appears probable, from the experiments described in a later section, that the metal present in the enzyme is firmly bound to the protein. It could not, however, be determined by dialysis whether all the metal found in the preparation was in stoichiometric combination, or whether some of it was derived from the reagents used during purification, since prolonged dialysis led to denaturation of the enzyme. If the zinc is assumed to be in stoichiometric amounts, the minimum molecular weight calculated for the enzyme is about 50000, which is one-third of that reported for muscle aldolase (Velick & Ronzoni, 1948).

Reaction balance. The conversion of 1 mole of FDP into 1 mole each of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate by *A. niger* aldolase was shown by the following

Table 2. *Equilibrium constant*

The test system contained 20 μ moles of tris, 150 μ g. of aldolase and the amount of FDP indicated below, in 1 ml. final volume at pH 7.6, temp. 30°.

Expt. no.	Initial FDP (μ moles)	Triose phosphates formed (μ moles)	Final FDP (calc.) (μ moles)	<i>K</i> (M)
1	5.8	2.0	4.8	2.08×10^{-4}
2	11.6	2.8	10.2	1.92×10^{-4}

experiments. The total amount of DPN reduced in the presence of a known amount of FDP was determined in the usual test system containing (a) 200 μ g. of *A. niger* aldolase and (b) the same quantity of aldolase and 100 μ g. of triose phosphate isomerase. In (a) 0.11 μ mole of FDP caused the reduction of 0.11 μ mole of DPN in the absence of isomerase and in (b) the same amount of FDP caused the reduction of 0.21 μ mole of DPN when isomerase was present. There was no DPN reduction when glyceraldehyde phosphate dehydrogenase, aldolase or arsenate was omitted from the test. The known high specificity of muscle glyceraldehyde phosphate dehydrogenase and isomerase show that the products of aldolase reaction on FDP were equivalent amounts of glyceraldehyde phosphate and dihydroxyacetone phosphate.

Equilibrium constant. The equilibrium constant for the reaction was determined by measuring the amount of total triose phosphate formed at 30° from known amounts of FDP after equilibrium had been attained. The total triose phosphates were measured as the increase in alkali-labile phosphorus (Herbert *et al.* 1940) and the amount of each of the triose phosphates present was taken to be half of the total since isomerase was absent. The quantity of enzyme taken was such that equilibrium was reached in 5 min., as shown by the fact that incubation for longer periods did not result in further increase in alkali-labile phosphorus. The value of the equilibrium constant *K*, which was calculated from

$$\frac{[\frac{1}{2} \text{ total triose phosphate}]^2}{[\text{residual FDP}]},$$

the concentrations being expressed in moles/litre, is shown in Table 2. The observed values are of the same order as those reported for muscle aldolase (Herbert *et al.* 1940) and pea aldolase (Stumpf, 1948).

Specificity. *A. niger* aldolase was found to be specific for FDP. Reduction of DPN was not observed in the usual test system containing 100 μ g. of aldolase and 100 μ g. of triosephosphate isomerase when glucose 6-phosphate, fructose 6-phosphate, fructose 1-phosphate, ribulose 5-phosphate or sedoheptulose 7-phosphate was used as substrate instead of FDP. The last three com-

Fig. 3. Effect of pH on enzyme activity.

pounds, if acted upon by aldolase, should form dihydroxyacetone phosphate and reduce DPN in the presence of triose phosphate isomerase and glyceraldehyde phosphate dehydrogenase.

Effect of enzyme concentration. The rate of DPN reduction was found to be proportional to enzyme concentration when the change in optical density/min. did not exceed 0.200. The amount of enzyme taken for the test was usually sufficient to cause a change of 0.010–0.080/min. Enzyme activity was also proportional to time when the total change in optical density was less than 0.600.

Effect of pH. The pH-activity curve for aldolase is shown in Fig. 3. Glyceraldehyde phosphate dehydrogenase, which has a markedly lower activity at lower pH values, was present in optimum amounts, since the activity was not increased by adding more of this enzyme. Precipitation of zinc occurred at higher pH values and the fall in activity above pH 8.0 was probably due to removal of metal from solution.

Effect of substrate concentration. The effect of substrate concentration on enzyme activity is shown in Fig. 4. The Michaelis constant for the reaction, K_m , was 1.5×10^{-3} M. K_m of rabbit muscle aldolase was found to be about 1×10^{-3} M (Herbert *et al.* 1940; Sibley & Lehninger, 1949).

Effect of temperature. The effect of temperature on enzyme activity is shown in Table 3. The enzyme, and not FDP, was added last in these tests, since there was considerable inactivation of

aldolase in the absence of substrate at temperatures greater than 30°. All the components of the test system, except aldolase, were incubated at the required temperature and the reaction was started by the addition of the enzyme which was at 0°, the change in temperature due to the small amount of enzyme being ignored. The relatively small increase in activity between 30° and 38° is probably due to inactivation of the enzyme.

Effect of inhibitors. The following compounds at the indicated final concentrations had no effect on aldolase activity: glucose (0.03 M), fructose (0.03 M), glucose 6-phosphate (0.016 M), fructose 6-phosphate (0.016 M), pyruvate (0.03 M), ribulose 5-phosphate (0.002 M), sedoheptulose 7-phosphate (0.005 M), arsenite (0.008 M), sulphanilamide (0.01 M), fluoride (0.01 M) and iodoacetate (0.01 M). The effect of iodoacetate, which inactivates triose phosphate dehydrogenase in the usual test, was determined by the method of Sibley & Lehninger (1949). Sulphanilamide was tested since it has been found to be inhibitory to carbonic anhydrase, which is also a zinc-containing enzyme (Mann & Keilin, 1940). The activity of aldolase was found to be the same in

tris, glycylglycine or veronal buffers at the same pH. The addition of 0.05 ml. of saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 7.0–8.5) resulted in an inhibition of aldolase, which varied from 5 to 15 % of the initial activity. The effect varied with different samples of $(\text{NH}_4)_2\text{SO}_4$ and was probably due to trace impurities since equivalent amounts of potassium sulphate and ammonium chloride were not inhibitory.

Effect of metal-binding agents. *A. niger* aldolase was found to be inhibited by metal-binding agents such as EDTA, *o*-phenanthroline, $\alpha\alpha'$ -dipyridyl and pyrophosphate. This inhibition was reversed by the addition of heavy metals. The effect of these inhibitors was studied in the presence of cysteine or GSH. These compounds were also inhibitory to aldolase so that the observed effect of EDTA, for instance, was due to the combined action of EDTA and cysteine or GSH. The sulphhydryl compounds were necessary in the test system for the activity of muscle glyceraldehyde phosphate dehydrogenase. The effect of EDTA on aldolase was markedly different when it was studied by a new method of aldolase estimation in the complete absence of cysteine, and will be discussed below.

Cysteine showed marked inhibition of aldolase in the presence of oxygen, when no zinc was added to the test system. The effect of cysteine was more complex under anaerobic conditions. There was an initial inhibition, which was much less than that observed aerobically, followed by a gradual increase in activity till the final value was equal to that obtained in the presence of added zinc. This reactivation was probably due to the presence of iron in cysteine (about 1 $\mu\text{g.}/60 \mu\text{moles}$ of cysteine). The metal is present in the oxidized state in air in the presence of cysteine and is reduced to the catalytically active bivalent ions anaerobically (Warburg & Christian, 1943). Inhibition was less at lower concentrations of cysteine and varied with different samples of cysteine. GSH was much less effective than cysteine as an inhibitor in the presence of oxygen and showed no inhibition under anaerobic conditions.

Fig. 4. Effect of substrate concentration $[S]$. V is expressed as $\Delta E/\text{min.}$ at 340 $m\mu$. $K_m = 1.5 \times 10^{-3} M$.

Table 3. *Effect of temperature on aldolase activity*

Activity determinations were carried out as described in the text, using 1.52 $\mu\text{g.}$ of aldolase in each test. The specific activities of crystalline muscle and yeast aldolases were calculated from the data in the references indicated in the table.

	Temp.	Change in optical density at 340 $m\mu$. ($\Delta E/\text{min.}$)	Specific activity (units/mg.)	Reference
<i>A. niger</i> aldolase	20°	0.053	17	—
	30°	0.123	39	—
	38°	0.153	49	—
Yeast aldolase	20°	—	17	(Warburg & Gawehn, 1954)
Rat-muscle aldolase	20°	—	14	(Warburg & Christian, 1943)
Rabbit-muscle aldolase	30°	—	28	(Baranowski & Niederland, 1949)

Table 4. *Effect of metal-binding agents on aldolase activity*

Composition of the assay system was the same as in the routine test except for Zn^{2+} , cysteine and EDTA. Zn^{2+} was not added except to the control. Cysteine or GSH and other inhibitors were added at the indicated levels.

Cysteine ($\times 10^{-2}\text{M}$)	GSH ($\times 10^{-2}\text{M}$)	EDTA ($\times 10^{-4}\text{M}$)	<i>o</i> -Phenan- throline ($\times 10^{-3}\text{M}$)	$\alpha\alpha'$ - Dipyridyl ($\times 10^{-3}\text{M}$)	Pyrophos- phate ($\times 10^{-3}\text{M}$)	Gas phase	Changes in optical density at 340 m μ . ($\Delta E/\text{min.}$)	Inhibition (%)
Control*	—	—	—	—	—	H_2 or O_2	0.080	—
0.2	—	—	—	—	—	H_2 or O_2	0.080	0
2.0	—	—	—	—	—	H_2	{ 0.038† 0.076†	53 5
2.0	—	—	—	—	—	O_2	0.017	79
2.0	—	1.8	—	—	—	H_2 or O_2	0	100
—	2.0	—	—	—	—	H_2	0.080	0
—	2.0	—	—	—	—	O_2	0.064	20
—	2.0	1.8	—	—	—	O_2	0.025	69
—	2.0	7.2	—	—	—	O_2	0.002	98
2.0	—	—	3.3	—	—	H_2	0.010	88
2.0	—	—	6.6	—	—	H_2	0.004	95
2.0	—	—	13.2	—	—	H_2	0.001	99
2.0	—	—	—	1.0	—	H_2	0.014	83
2.0	—	—	—	10.0	—	H_2	0.010	88
0.2	—	—	—	—	3.3	H_2	0.062	23
0.2	—	—	—	—	26.0	H_2	0.004	95
2.0	—	—	—	—	1.0	H_2	0.010	88
2.0	—	—	—	—	3.3	H_2	0.002	98

* Activity was determined as in the routine test.

† Change in E between the sixth and eighth minutes.

† Change in E during the first 2 min.

The effect of EDTA, *o*-phenanthroline, dipyrldyl and pyrophosphate on aldolase is shown in Table 4. EDTA was the most effective inhibitor, and at a concentration of $1.8 \times 10^{-4}\text{M}$ (in the presence of 0.02M cysteine) completely suppressed aldolase activity. The enzyme was completely inhibited immediately after the addition of EDTA and the extent and rapidity of inhibition were the same whether the enzyme, EDTA or FDP was added last to the test system (cf. hydrazine test described below). The other inhibitors showed marked inhibition only at higher concentrations and did not completely inhibit aldolase activity in the concentration range which was studied. At lower cysteine concentrations or with GSH the effect of all these inhibitors was less pronounced, indicating that the observed inhibition was due to the effect of both cysteine and the other complexing agent. Yeast aldolase was reported to be irreversibly inhibited by pyrophosphate (Warburg & Christian, 1943). Inhibition of *A. niger* aldolase by pyrophosphate, as well as by the other inhibitors, was completely reversible. Aldolase activity was fully restored by $2.7 \times 10^{-4}\text{M}$ - Zn^{2+} , even when the enzyme was initially incubated with 0.1M -pyrophosphate at pH 8.0 for 10 min. at 0° or 38° before addition to the test system (Table 5).

Effect of metals. Aldolase was fully active in the absence of added metals. At low concentrations of cysteine or GSH (0.002M) the activity was the same with or without added zinc. The effect of

metals was therefore studied with enzyme which was inhibited by cysteine, EDTA or *o*-phenanthroline. Solutions of the respective metal salts were prepared immediately before use. Controls, without enzyme and without substrate respectively, were run and the blanks, if any, were subtracted from the experimental values. When Fe^{2+} or Co^{2+} was added to solutions containing EDTA or cysteine, the extinction of the solutions changed rapidly during the first few minutes and then remained nearly constant. The reaction was started by the addition of FDP when the readings were nearly constant in order to minimize the blanks. The activities obtained in all the experiments described below were found to be proportional to enzyme concentration and time within the limits previously indicated.

Inhibition by EDTA was reversed by Zn^{2+} , Mn^{2+} , Fe^{2+} , or Co^{2+} but not by Mg^{2+} , Ca^{2+} , Cu^{2+} or MoO_4^{2-} (Table 5). (Some enzyme preparations, especially those of low purity, showed a slightly higher activity with EDTA and Zn^{2+} than with Zn^{2+} alone, possibly due to the chelation of trace-metal impurities by EDTA. Routine assay of the enzyme was therefore carried out with EDTA and Zn^{2+} in the test system.) Zinc was active both under aerobic and anaerobic conditions, whereas iron and cobalt were effective only in the absence of oxygen. Ferrous and cobaltous ions are oxidized to the tervalent state by oxygen in the presence of cysteine. Ferric ions can be reduced again to the

ferrous state by cysteine in the absence of oxygen, whereas the oxidation of cobalt to the cobaltic state is irreversible. This is shown in Table 5. When Co^{3+} was mixed with the test solution containing cysteine in air and the air was then replaced by H_2 there was no aldolase activity. But when cysteine and Co^{2+} were mixed under H_2 , the enzyme was reactivated. Aldolase activity was obtained with Fe^{2+} whether it was mixed with cysteine in air or H_2 .

Manganous salts activated aldolase in the presence or absence of oxygen, though the activity was about 40 % less under aerobic conditions. The effect of the metal was not however studied in further detail. The failure of cupric ions to reverse EDTA inhibition was not due to inhibition of glyceraldehyde phosphate dehydrogenase since aldolase activity was observed when both Cu^{2+} and Zn^{2+} were added to the test solution. Reversal of *o*-phenanthroline inhibition by Zn^{2+} or Mn^{2+} , and reversal of pyrophosphate or cysteine inhibition by Zn^{2+} are shown in Table 5. The effect of other metals with these inhibitors could not be studied owing to the formation of precipitates or highly coloured complexes with high light absorption at

340 $\text{m}\mu$. The amount of metal required to counteract the effect of EDTA was slightly in excess of the stoichiometric quantity, whereas much smaller amounts sufficed to reverse inhibition by pyrophosphate or phenanthroline.

Spectrophotometric assay of aldolase with hydrazine. The inhibition of aldolase by EDTA and reactivation by Zn^{2+} were confirmed by a new spectrophotometric method for the estimation of aldolase activity. When aldolase and FDP were mixed together in the presence of small amounts of hydrazine, the rate of increase in the optical density of the solution at 240 $\text{m}\mu$. was found to be proportional to enzyme concentration. The experimental conditions are shown in Fig. 5. Hydrazine could be replaced by semicarbazide but not by hydroxylamine. The method is comparable in sensitivity to the optical test of Warburg & Christian (1941). The hydrazine test has the advantage that it can be used in the presence of enzymes which oxidize or destroy reduced DPN, and interfere with the other method. The test system is also less complex and contains only the substrate and hydrazine, the buffer being unnecessary if the other reagents are adjusted to the

Table 5. *Reactivation of aldolase by metals*

Reaction mixture for assay of enzyme activity contained all the components of the routine test system except cysteine, EDTA and Zn. Other additions are shown below.

Cysteine ($\times 10^{-2}\text{M}$)	EDTA ($\times 10^{-4}\text{M}$)	<i>o</i> -Phenanthroline ($\times 10^{-3}\text{M}$)	Pyrophosphate ($\times 10^{-2}\text{M}$)	Metal	Concn. of metal ($\times 10^{-4}\text{M}$)	Gas phase	Change in optical density at 340 $\text{m}\mu$. ($\Delta E/\text{min.}$)
0.2	—	—	—	—	—	H_2 or O_2	0.082
0.2	—	—	—	Zn^{2+}	2.7	H_2 or O_2	0.080
2.0	—	—	—	—	—	O_2	0.017
2.0	—	—	—	Zn^{2+}	2.7	O_2	0.078
2.0	1.8	—	—	—	—	O_2 or H_2	0
2.0	1.8	—	—	Zn^{2+}	2.7	O_2 or H_2	0.082
2.0	1.8	—	—	Mn^{2+}	2.7	H_2	0.068
2.0	1.8	—	—	Mn^{2+}	2.7	O_2	0.036
2.0	1.8	—	—	Mn^{2+}	4.0	H_2	0.075
2.0	1.8	—	—	Fe^{2+}	2.7	H_2	0.065
2.0	1.8	—	—	Fe^{2+}	4.0	H_2	0.075
2.0	1.8	—	—	* Fe^{2+}	4.0	H_2	0.077
2.0	1.8	—	—	Co^{2+}	2.7	H_2	0.078
2.0	1.8	—	—	* Co^{2+}	2.7	H_2	0
2.0	1.8	—	—	{ Co^{2+} , Zn^{2+} }	{2.7 2.7}	H_2	0.078
2.0	1.8	—	—	Mg^{2+} or Ca^{2+} or MoO_4^{2-}	2.7 or 4.0	H_2	0
2.0	1.8	—	—	Cu^{2+}	2.7 or 4.0	H_2	0
2.0	1.8	—	—	{ Cu^{2+} , Zn^{2+} }	{2.7 2.7}	H_2	0.076
2.0	—	2.0	—	—	—	H_2	0
2.0	—	2.0	—	Zn^{2+}	2.7	H_2	0.082
2.0	—	2.0	—	Mn^{2+}	2.7	H_2	0.056
2.0	—	—	3.0	—	—	H_2	0.002
2.0	—	—	3.0	Zn^{2+}	2.7	H_2	0.074
2.0	—	—	3.0†	Zn^{2+}	2.7	H_2	0.070

* Solution of metal salt was mixed with cysteine in air, and air was then replaced by H_2 .

† Aldolase was incubated with 0.1M pyrophosphate of pH 7.6, at 0° or 30° for 10 min. before being tested.

required pH. The method has, however, the disadvantage that the effect of substances having high light absorption at $240\text{ m}\mu$. (Fe^{2+} , Co^{2+} , *o*-phenanthroline, etc.) cannot be studied. An appreciable non-enzymic blank was also obtained when FDP and hydrazine were mixed together, but the blank was relatively low when the concentration of hydrazine was reduced to the level indicated in the legend to Fig. 5. There was a rapid initial change in the $240\text{ m}\mu$. reading when FDP and hydrazine were mixed, probably due to impurities in the FDP since there was only a small but constant change after 2–3 min. The readings obtained with enzyme were corrected for this blank. The amount of enzyme taken for the test was such that the rate of change of $E(\Delta E)/\text{min.}$ was less than 0.150. ΔE showed a slight increase during the first 2 or 3 min. after the addition of aldolase and then reached a constant value which was proportional to enzyme concentration. This method can be used for the determination of the aldolase activity of crude extracts or of purified enzyme and gave results comparable to those obtained by other methods. It has, however, been used primarily to determine the effect of EDTA and Zn^{2+} on aldolase in a system which does not contain cysteine, DPN, glyceraldehyde phosphate dehydrogenase, etc. The high value of E and the rapidity of formation of the hydrazones of other compounds (e.g. glyoxylic acid) suggests that spectrophotometric

methods with hydrazine as trapping agent can be extended to other enzyme reactions in which aldehydes or ketones are formed.

Fig. 6 shows inhibition of aldolase by EDTA and reversal of inhibition by Zn^{2+} , confirming the results obtained by the other method. But in the hydrazine test EDTA was ineffective at the levels used in the routine test system. Even at higher concentrations of the inhibitor, enzyme activity decreased slowly with time and became negligible only after 10 min. or more. Similar results were obtained when EDTA, FDP and hydrazine were incubated for 10–20 min. before the addition of enzyme. But when the enzyme and EDTA were incubated together for 10 min. and then added to the other components of the test, the enzyme was completely inhibited from the beginning of the reaction. These experiments indicate that chelation

Fig. 5. Hydrazine test: effect of enzyme concentration on activity. The test solution containing 0.5 ml. of 0.2M tris, $7\mu\text{mole}$ of hydrazine and $12\mu\text{mole}$ of FDP was incubated for 5 min. before adding the enzyme. Final vol.: 3 ml.; pH 7.6, temp. 30° . E at $240\text{ m}\mu$. was corrected for the blank.

Fig. 6. Hydrazine test: effect of EDTA and Zn on aldolase activity. The test solution containing 0.5 ml. of 0.2M tris, $7\mu\text{mole}$ of hydrazine and $12\mu\text{mole}$ of FDP was incubated for 5 min. Enzyme was added last. Other additions are indicated below. Final volume: 3 ml.; pH 7.6, temp. 30° . E at $240\text{ m}\mu$. was corrected for the blank. Curve 1. $\circ$, Enzyme incubated for 10 min. in $1.4 \times 10^{-3}\text{M}$ EDTA before adding to the test system; final EDTA concn. $3.6 \times 10^{-4}\text{M}$. $\downarrow$, Zn^{2+} was added to a final concn. of $4.0 \times 10^{-4}\text{M}$. Curve 2. $\bullet$, EDTA $2.7 \times 10^{-3}\text{M}$. $\downarrow$, Zn^{2+} was added to a final concn. of $3.0 \times 10^{-3}\text{M}$. Curve 3. $\blacktriangle$, EDTA $3.6 \times 10^{-4}\text{M}$. $\downarrow$, Zn^{2+} was added to a final concn. of $4.0 \times 10^{-4}\text{M}$. Curve 4. $\circ$, No addition. Curve 5. $\triangle$, Zn^{2+} $1.8 \times 10^{-4}\text{M}$. In curves 2 and 3, EDTA was incubated with the other components of the test solution for 10 min. before addition of the enzyme.

of the metal present in the enzyme, and not in the other reagents, by EDTA is necessary for inhibition of activity. Reversal of EDTA inhibition by Zn^{2+} was very rapid, showing that the rate-limiting step during inhibition is the combination of EDTA with the enzyme-linked metal and not with free Zn^{2+} . The rapid inhibition observed in the other method of aldolase assay was probably due to the combined action of cysteine and EDTA, which could not be studied in the hydrazine test owing to high light absorption at 240 μ .

'Aldolase inhibitor'. It has been suggested that the metal requirement of yeast aldolase is due to the presence of an aldolase inhibitor, the effect of which can be reversed by heavy metals (Meyerhof, 1951). Several fractions obtained during the purification of aldolase, crude extracts and boiled extracts of *A. niger* were tested for inhibitory action on aldolase from muscle, *A. niger* or peas, either directly or in the presence of EDTA or zinc. They were also tested for aldolase activity in the presence of EDTA, since any fraction containing more aldolase than aldolase inhibitor should be active even in the presence of EDTA.

These experiments need not be described in detail since all of them yielded negative results. The electrophoretic experiments also showed no indication of a second component in pure aldolase. Though these results do not necessarily disprove the existence of an aldolase inhibitor in *A. niger*, especially if firmly bound to aldolase, the hypothesis remains at present without any experimental basis.

DISCUSSION

The inhibition of pure *A. niger* aldolase by metal-binding agents and its reactivation by zinc and other metals show that the mould enzyme differs from muscle aldolase and resembles the aldolases of yeast and *Cl. perfringens* in requiring heavy metals for its activity. The metallo-aldolases also show marked differences in metal specificity and in other properties. The bacterial enzyme was activated only by Fe^{2+} or Co^{2+} but not by Mn^{2+} or Zn^{2+} , and after ageing the enzyme required both cysteine and metal for maximum activity (Bard & Gunsalus, 1950). The aldolases of yeast and *A. niger* show a marked resemblance in several properties such as reversible inhibition by cysteine, *o*-phenanthroline and dipyriddy, activation by Zn^{2+} , Fe^{2+} or Co^{2+} , inhibition in the presence of cysteine and Fe^{2+} (or Co^{2+}) by oxygen, etc. But the two enzymes differ in other respects. Yeast aldolase has been reported to be activated by Cu^{2+} but not by Mn^{2+} , whereas *A. niger* aldolase was activated by Mn^{2+} but not by Cu^{2+} . Moreover, pyrophosphate inhibition of *A. niger*, unlike that of the yeast enzyme, was completely reversible by Zn^{2+} .

A. niger aldolase appears to show a broad

specificity in its metal requirement, but the observed effects of Zn^{2+} , Mn^{2+} , Fe^{2+} and Co^{2+} were due to the reversal of inhibition by metal-binding agents. This is not as direct a proof of metal specificity as a demonstration of activation by metals in the absence of the inhibitors, since the action of the added metal can also be due to combination with the inhibitor, thereby releasing the metal which was originally bound to the enzyme. Inhibition by EDTA could not, however, be reversed by Mg^{2+} , Ca^{2+} or Cu^{2+} over a wide concentration range, though these metals are known to form complexes with the chelating agent. The concentration of metal required for reversing the effect of dipyriddy, *o*-phenanthroline and pyrophosphate was only a small fraction of that required for stoichiometric combination with these inhibitors. It appears probable, though not certain, that the effect of Zn^{2+} , Mn^{2+} , Fe^{2+} and Co^{2+} is due to activation of aldolase.

The nature of the difference between muscle aldolase and the metal-requiring aldolases, and the function of the metal in the latter, remain obscure. The stability of *A. niger* aldolase was not dependent on the presence of metals. The hypothesis of an 'aldolase inhibitor' could not be substantiated. Aldolases which can utilize fructose 1-phosphate as substrate have been recently reported to be present in liver (Leuthardt, Testa & Wolf, 1953) and in jack-beans (Cardini, 1952). But *A. niger* aldolase does not belong to this group of enzymes, since it resembles muscle aldolase in having a negligible action on fructose 1-phosphate. It is also improbable that the reactions catalysed by muscle and *A. niger* aldolase are different in any respect, except in metal requirement, as will be evident from the data on reaction balance, Michaelis constant, equilibrium constant and temperature coefficient of activity. A possible explanation for the metal requirement of the aldolases of *A. niger*, yeast, etc., is that the catalytically active molecule of these enzymes, unlike that of muscle aldolase, consists of two or more inactive smaller molecules, which are firmly but reversibly linked together by metal atoms. The experiments described in the section on the hydrazine test show that the enzyme-bound metal is not present in a loosely adsorbed form or in ionic combination. The differences in the metal specificity of the metallo-aldolase from different sources and the activation of each individual enzyme by several different metals suggest a relatively non-specific role for the metal, unlike that, for instance, in the iron-porphyrin enzymes. The zinc content of *A. niger* aldolase indicates a lower minimum molecular weight than that of muscle aldolase. It was unfortunately not possible to determine the actual molecular weight of the purified enzyme by

physical methods. A study of the effect of EDTA on the molecular weight of the enzyme to determine whether the removal of the metal present in the enzyme results in a lowering of the molecular weight should be of value in testing the validity of this hypothesis.

SUMMARY

1. The aldolase of *Aspergillus niger* has been purified by ammonium sulphate fractionation and adsorption on calcium phosphate gel.

2. The purified aldolase was electrophoretically homogeneous. The activity per milligram of the enzyme was equal to that of crystalline muscle or yeast aldolase.

3. The properties of the enzyme and the kinetics of the reaction are described.

4. The enzyme resembled yeast aldolase and differed from muscle aldolase in requiring heavy metals for its activity. It was inhibited by cysteine, pyrophosphate, ethylenediaminetetraacetic acid, *o*-phenanthroline and $\alpha\alpha'$ -dipyridyl. The inhibition was reversed by bivalent zinc, manganese, iron or cobalt but not by magnesium, calcium or copper.

5. A new spectrophotometric method for the estimation of aldolase activity is described. The method is based on the increase in light absorption at 240 m μ . in the presence of hydrazine.

6. The differences between the aldolases from different tissues are briefly discussed.

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A Specific Polysaccharide of *Pasteurella pestis*

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When many strains of *Pasteurella pestis* are grown at 37° a gelatinous envelope surrounds the cells (Rowland, 1914); this envelope material will immunize mice against plague infection (Amies, 1951; Baker, Sommer, Foster, Meyer & Meyer, 1952). Schütze (1932) showed that in addition to the envelope antigen, which is heat-labile, a heat-stable somatic antigen is present in rough and

smooth forms of *Past. pestis* and is common to the closely related rodent-pathogen *Pasteurella pseudotuberculosis*. It was considered that although a smooth form of colony is characteristic of most *Past. pestis* strains, including all the virulent ones, no smooth 'O' somatic antigen was present, but that the heat-stable antigen was a rough somatic antigen underlying the envelope.