Purification and Characterization of Two Fructose Diphosphate Aldolases from *Escherichia coli* (Crookes' Strain)

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Two fructose diphosphate aldolases (EC 4.1.2.13) were detected in extracts of *Escherichia coli* (Crookes' strain) grown on pyruvate or lactate. The two enzymes can be resolved by chromatography on DEAE-cellulose at pH 7.5, or by gel filtration on Sephadex G-200, and both have been obtained in a pure state. One is a typical bacterial aldolase (class II) in that it is strongly inhibited by metal-chelating agents and is reactivated by bivalent metal ions, e.g. Ca^{2+}, Zn^{2+}. It is a dimer with a molecular weight of approx. 70000, and the K_m value for fructose diphosphate is about 0.85 mM. The other aldolase is not dependent on metal ions for its activity, but is inhibited by reduction with $NaBH_4$ in the presence of substrate. The K_m value for fructose diphosphate is about 20 μM (although the Lineweaver–Burk plot is not linear) and the enzyme is probably a tetramer with molecular weight approx. 140000. It has been crystallized. On the basis of these properties it is tentatively assigned to class I. The appearance of a class I aldolase in bacteria was unexpected, and its synthesis in *E. coli* is apparently favoured by conditions of gluconeogenesis. Only aldolase of class II was found in *E. coli* that had been grown on glucose. The significance of these results for the evolution of fructose diphosphate aldolases is briefly discussed.

Fructose 1,6-diphosphate aldolases (fructose 1,6-diphosphate D-glyceraldehyde 3-phosphate-lyases, EC 4.1.2.13) are divisible into two distinct classes on the basis of their molecular and catalytic properties [for reviews see Rutter (1964) and Morse & Horecker (1968)]. The primary distinction is in the mechanism of the aldol cleavage. Aldolases of class I, which are found in animals, plants and green algae, form a Schiff base between the ε-amino group of a specific lysine residue in the active site and the carbonyl group of the substrate. This imine may be reduced by borohydride, which therefore irreversibly inhibits class I aldolases in the presence of substrate. On the other hand, aldolases of class II, which are found in yeast, bacteria, fungi and blue–green algae, are insensitive to reduction with borohydride in the presence of substrate but are strongly inhibited by metal-chelating agents such as EDTA. The metal ion in the active site varies, but several metals including zinc, cobalt, iron, nickel and manganese can form an active metalloprotein complex (Kobes *et al.*, 1969). An intriguing situation is found in *Euglena gracilis*, *Chlamydomonas mundana* and *Chlamydomonas rheinhardii* which, depending on growth conditions, produce either a class I or a class II aldolase (Cremona, 1968; Russell & Gibbs, 1967; Guerrini *et al.*, 1971).

These differences between class I and class II aldolases are reflected in their characteristic molecular and kinetic properties. For example, class I aldolases are typically tetrameric (subunit mol.wt. approx.

40000), whereas class II aldolases are normally dimeric (subunit mol.wt. between 30000 and 40000) (Rutter, 1965). Similarly, class I aldolases have a K_m value for fructose diphosphate of approx. 5 μM (Rutter, 1965; Morse & Horecker, 1968), but for aldolases of class II the K_m rises to about 300 μM (Rutter *et al.*, 1966). The two classes of aldolase also differ in their sensitivity to digestion by carboxypeptidase, in their reaction with alkylating agents and in their pH profiles for cleavage of fructose diphosphate and exchange of ³H into dihydroxyacetone phosphate (Rutter, 1964). Taking note of these facts, Rutter (1964) has proposed that the two classes of aldolase have evolved independently from separate genetic origins, but that within each class the enzymes from different species are homologous. Amino acid sequence analysis of class I aldolases from a variety of sources (Gibbons *et al.*, 1970; Guha *et al.*, 1971) and of the class II aldolase from yeast (Jack & Harris, 1971) supports this view.

The present paper describes how, during the purification of the class II aldolase from *Escherichia coli* (Crookes' strain), a second type of aldolase was discovered. The new enzyme (aldolase 1) may be distinguished from the usual class II aldolase (aldolase 2) by virtue of its different molecular and catalytic properties, which would appear to place it in class I. The occurrence of such an aldolase in bacteria was unexpected in the light of the phylogenetic distribution of aldolases previously described. Its production in

E. coli is dependent on the growth conditions of the organism.

Materials and Methods

Materials

All reagents used were of A.R. grade. DEAE-cellulose was purchased as precycled DE-52 from H. Reeve Angel and Co. Ltd., London E.C.4, U.K., and Sephadex G-25, G-150 and G-200 were obtained from Pharmacia (G.B.) Ltd., London W.13, U.K. Hydroxyapatite was from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Fructose 1,6-diphosphate (tetrasodium salt) was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., and assayed by using rabbit muscle aldolase (Blostein & Rutter, 1963). Fructose 1-phosphate (disodium salt), an α-glycerol phosphate dehydrogenase–triose phosphate isomerase mixture and other crystalline enzymes were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Dihydroxy-acetone phosphate was prepared as the calcium salt from fructose 1,6-diphosphate and assayed enzymically by using α-glycerol phosphate dehydrogenase (Horecker *et al.*, 1963). Protamine sulphate (salmon testis) was purchased from Koch–Light Ltd., Colnbrook, Bucks., U.K., and NaBCNH$_3$ from R. N. Emanuel Ltd., Wembley, Middx., U.K.

Methods

Preparation of crude cell extracts. Cultures of *E. coli* (Crookes' strain) were grown in a basal salts medium on a variety of different carbon sources at the Microbiological Research Establishment, Porton Down, Wilts., U.K. In most cases lactate was the carbon source, but in some experiments glucose or pyruvate was used. Batches of up to 4 kg of cell paste were disrupted in 20 mM-sodium phosphate buffer, pH 7.0, with a Manton–Galling homogenizer. The same quality of extract could be obtained on a smaller scale by sonicating for 4 min at 50 kHz in a Mullard sonicator. During the disruption of the cells the temperature did not rise above 20°C. The homogenate was centrifuged at 15000 g and 2°C for 1 h to remove cell debris and the pH of the supernatant was adjusted to pH 6.2 with 1% (v/v) acetic acid. Up to 0.3 vol. of 2% (w/v) protamine sulphate, pH 6.2, was then added in a stepwise manner, the precipitate which formed at each step being removed by centrifugation at 2°C and retained for subsequent purification of the α-oxo acid dehydrogenase multi-enzyme complexes (Reed & Mukherjee, 1969; Perham & Thomas, 1971). This preparation process, which was carried out by Dr. K. Sargeant and Mr. A. R. Whitaker at the Microbiological Research Establishment, Porton, left all the aldolase activity in the supernatant fraction, and this was used as the starting material for the purification of the enzymes.

Enzyme assays. Two different assays for fructose diphosphate aldolase activity were employed, a modification of the colorimetric assay with Dnp-hydrazine (Sibley & Lehninger, 1949) and a coupled spectrophotometric assay (Blostein & Rutter, 1963). The colorimetric assay was used to assay column eluates and in certain cases where the presence of additional reagents interfered with the coupling enzymes in the coupled assay. It was modified to allow the incubation of enzyme with substrate and the subsequent assay of hydrazones so formed to be carried out in the same test tube, thereby obviating the need for sampling and saving time and materials. The standard assay mixture contained 0.05 ml of 0.56 M-hydrazine hydrochloride adjusted to pH 7.5 with solid Tris, 0.05 ml of 50 mM-fructose diphosphate in 50 mM-Tris–HCl buffer, pH 7.5, and 0.35 ml of de-ionized water containing additions of thiols, metal ions or chelating agents if required. The reaction was started by adding enzyme (approx. 20 μg). Incubation was at 37°C for 20 min and the reaction was stopped by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid. The hydrazones formed during the incubation were assayed in the same tube by adding, in the order given, 1 ml of 0.75 M-NaOH and incubating at 20°C for 10 min, 1 ml of Dnp-hydrazine (1 mg/ml in 2 M-HCl) and incubating at 37°C for 10 min, followed by 7 ml of 0.75 M-NaOH. The absorbance at 540 nm was measured in a Unicam SP.500 spectrophotometer against a complete reagent blank; the colour was stable in the interval 3–15 min after addition of the last reagent.

The coupled assay (Blostein & Rutter, 1963) was carried out at 30°C in a 3 ml final volume of 50 mM-Tris–HCl buffer, pH 7.5, containing 6 μg of glycerol phosphate dehydrogenase and triose phosphate isomerase, 0.6 μmol of NADH and 9 μmol of fructose diphosphate. The reaction was started by the addition of enzyme and the decrease in the absorbance at 340 nm was followed with a Gilford 2000 spectrophotometer.

Determination of subunit molecular weight. Proteins were reduced and *S*-carboxymethylated in 5 M-guanidine–HCl as described by Gibbons & Perham (1970). Samples of protein were run in 7.5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate (Shapiro & Maizel, 1969; Weber & Osborn, 1969). The markers used for the estimation of molecular weight were bovine serum albumin (mol.wt. 68000), pig muscle glyceraldehyde 3-phosphate dehydrogenase (subunit mol.wt. 36000) and hen's-egg-white lysozyme (mol.wt. 12300). A linear plot for log (mol.wt.) against electrophoretic mobility was obtained.

Measurement of protein concentration. The protein concentration in solutions was measured spectro-photometrically by the method of Warburg &

Christian (1941). Close agreement with the dry weight of desalted proteins was found.

Electroconductivity measurements. The equilibration of ion-exchange columns and the progress of salt gradients were followed by measurements of electroconductivity with an MEL conductivity probe (Pye Unicam, Cambridge, U.K.).

Amino acid analysis. Samples of reduced carboxymethylated proteins were hydrolysed with 6M-HCl in sealed, evacuated tubes for 24h at 105°C and analysed in duplicate for amino acids by using a Beckman 120C automatic analyser as described by Perham (1967).

Results

Purification of aldolases 1 and 2

All steps in the purification were carried out at room temperature (20°C), since experience showed that there was no advantage in working at a lower temperature. In preliminary experiments the protamine sulphate supernatant derived from *E. coli* grown on pyruvate was dialysed against 50mM-Tris-HCl buffer, pH7.5, and applied to a column of DEAE-cellulose equilibrated with the same buffer. All the aldolase activity was retained by the column. A linear gradient of NaCl from 0-250mM in the same 50mM-Tris-HCl buffer, pH7.5, eluted two separate peaks of aldolase activity. The first peak was eluted at approx. 50mM-Tris-HCl buffer-20mM-NaCl, whereas the second peak was eluted with 50mM-Tris-HCl buffer-75mM-NaCl. Each peak was rechromatographed separately on the same column and continued to run as a single peak at the same position on the gradient. The two peaks of activity could not be interconverted by treatment with chelating agents, metal ions or thiol reagents. They were labelled aldolase 1 and aldolase 2 in the order of their elution from the DEAE-cellulose column.

The relative amounts of the two aldolases present in cell-free extracts of *E. coli* grown on different carbon sources were investigated. The enzymes in small samples of extract were separated by chromatography on DEAE-cellulose by using the gradient system described above and the relative activities of the two peaks were estimated. The exact proportions of the two enzymes present *in vivo* are somewhat difficult to assess because aldolase 2 is less stable than aldolase 1. However, of the total aldolase activity in cell-free extracts of *E. coli* grown on pyruvate and lactate, approx. 60% was accounted for by aldolase 1. On the other hand, *E. coli* grown on glucose showed no
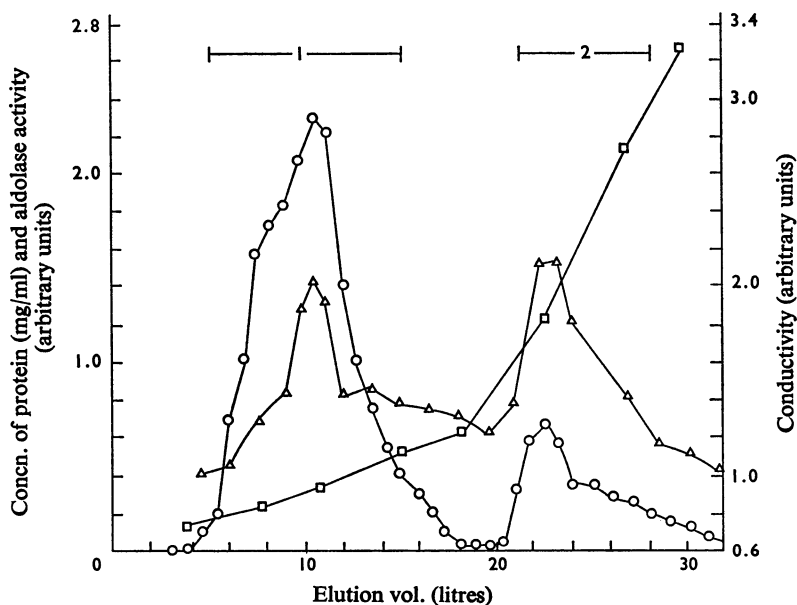

Fig. 1. *Chromatography of aldolases from E. coli on DEAE-cellulose*

The protamine sulphate supernatant from cell-free extracts of *E. coli* grown on lactate was adsorbed on a column (34cm×9cm) of DEAE-cellulose, which was developed with two successive gradients of NaCl in 50mM-Tris-HCl buffer, pH7.5 (see the text). Aldolase 1 and aldolase 2 are labelled 1 and 2 respectively. ○, Aldolase activity (arbitrary units); △, protein concentration (mg/ml); □, conductivity (arbitrary units).

aldolase 1 activity, but had an increased aldolase 2 activity. For these experiments the coupled assay was used. *E. coli* cells that had been grown on lactate were used for the large-scale purification of aldolase 1 and aldolase 2.

The protamine sulphate supernatant derived from up to 2 kg of cell paste was adjusted to pH 7.5 with 1 M-Tris and diluted to the same electroconductivity as the 50 mM-Tris–HCl buffer, pH 7.5. The supernatant was then applied to a column (34 cm × 9 cm; one column volume is approx. 2 litres) of DEAE-cellulose equilibrated with 50 mM-Tris–HCl buffer, pH 7.5, and the protein that was not adsorbed and which contained no aldolase activity was discarded. The column was washed with 2 column vol. of 50 mM-Tris–HCl buffer, pH 7.5, followed by a linear gradient of 0–50 mM-NaCl in the same buffer, over a total of 10 column volumes. The flow rate was 30 ml·cm^{-2}·h^{-1}. When the first gradient ended, a second was applied, 50–250 mM-NaCl over the same volume, in the same buffer. Fractions (50 ml) were collected and monitored for protein concentration, pH, conductivity and aldolase activity. The fractions with high specific activity for aldolases 1 and 2 were pooled separately, as shown in Fig. 1.

The pooled fractions containing aldolase 1 were diluted with 2 vol. of deionized water and applied to a column (15 cm × 6 cm) of DEAE-cellulose equilibrated with 50 mM-Tris–HCl buffer, pH 7.5. Flow rates as high as 100 ml·cm^{-2}·h^{-1} were used with total retention of the aldolase activity. The aldolase activity was eluted in a small volume by 50 mM-Tris–HCl buffer–200 mM-NaCl, pH 7.5. The protein solution obtained was then fractionated by stepwise addition of solid $(NH_4)_2SO_4$. The fraction precipitating between 1.9 and 2.6 M-$(NH_4)_2SO_4$ contained 90% of the aldolase activity and this precipitate, collected by centrifugation, was redissolved in a minimal amount of buffer. Finely powdered $(NH_4)_2SO_4$ was added to the aldolase solution until it was slightly cloudy. After the aldolase solution was left overnight at 2°C, crystals formed and increased in amount over the next few days. Further small additions of $(NH_4)_2SO_4$ assisted the complete crystallization of aldolase 1. An overall yield of about 50% gave 150 mg of crystalline aldolase (specific activity 1 μmol of fructose 1,6-diphosphate/min per mg) from 2 kg of cell paste.

The pooled fractions containing aldolase 2 from the large DEAE-cellulose column, after incubation for 12 h with 1 mM-ZnCl$_2$ at 2°C, were diluted threefold and then concentrated on to a column (12 cm × 4 cm) of DEAE-cellulose equilibrated with 50 mM-Tris–HCl buffer, pH 7.5. A linear gradient of 15 column volumes of 50–200 mM-NaCl in the same buffer gave a sharp peak of aldolase activity in the same position on the gradient for aldolase 2 as before. The fractions (15 ml) with specific activity greater than 75% of the maximum were pooled and the pH adjusted to 6.8 with 0.1 M-HCl. The enzyme was further purified by adsorption on calcium phosphate gel (Keilin & Hartree, 1938). Gel was added to the aldolase solution (1–2 mg/ml) in an amount equivalent to 0.5 mg dry weight of gel to 1 mg of protein. After stirring gently for 30 min the gel with adsorbed aldolase was collected by low-speed centrifugation, washed once with deionized water and then with three successive washes of 0.4 M-sodium phosphate–0.4 M-$(NH_4)_2SO_4$, pH 7.5. The last three washes, when pooled, contained 90% of the aldolase 2 activity eluted from the DEAE-cellulose. The pooled washings were fractionated by stepwise addition of solid $(NH_4)_2SO_4$; the fraction that was precipitated between 2.25 and 3.0 M-$(NH_4)_2SO_4$ contained 80% of the aldolase activity. The $(NH_4)_2SO_4$ precipitate, collected by centrifugation, was redissolved (about 20 mg/ml) in 10 mM-sodium phosphate buffer, pH 6.8, and dialysed against the same buffer to remove the $(NH_4)_2SO_4$. The aldolase was applied to a column (10 cm × 3 cm) consisting of a 1:1 (w/w) mixture of hydroxyapatite and cellulose fibres selected to remove fines. A linear gradient (20 column volumes) from 10 mM-sodium phosphate buffer, pH 6.8, to 150 mM-sodium phosphate buffer, pH 6.8, eluted the aldolase as a sharp peak at about 75 mM-sodium phosphate buffer. The fractions with specific activity greater than 80% of the maximum were pooled and precipitated with solid $(NH_4)_2SO_4$ (600 g/l). The precipitate, collected by centrifugation, was fractionated further by resuspending it in $(NH_4)_2SO_4$ solutions of gradually decreasing concentration. The insoluble material at each wash was removed by centrifugation and resuspended. The aldolase redissolved when the $(NH_4)_2SO_4$ concentration had fallen to 2.1–2.3 M. The yield (approx. 25%) of aldolase 2 from 2 kg of *E. coli* cell paste was approx. 50 mg.

Characterization of aldolases 1 and 2

Molecular-weight determinations. After reduction and *S*-carboxymethylation, both aldolases ran on sodium dodecyl sulphate–polyacrylamide gels as single bands with a mobility slightly greater than that of the subunits of rabbit glyceraldehyde 3-phosphate dehydrogenase (subunit mol.wt. 36000). A mixed sample of aldolases 1 and 2 ran as a single band with an estimated mol.wt. 34500±500. Aldolase 2 that had not been reduced and carboxymethylated ran on gels as two bands with molecular weights of 70000 and 35000, suggesting dimeric and monomeric forms. This effect was not seen with aldolase 1.

The behaviour of native aldolase 1 and aldolase 2 during gel filtration on columns of Sephadex G-150 and G-200 in 50 mM-Tris–HCl buffer, pH 7.5, was compared with that of known protein markers. The elution position on these gel-filtration columns, taken

Table 1. *Amino acid composition of aldolases 1 and 2*

S-Carboxymethylated protein was hydrolysed for 24h in 6M-HCl at 105°C. Amino acid analyses were carried out with a Beckman 120C autoanalyser. A subunit molecular weight of 34500 was assumed for both enzymes and the results are the mean of two separate analyses. Abbreviation: CmCys, *S*-carboxymethylcysteine.

| Amino acid	Composition (residues/34500 daltons)	
	Aldolase 1	Aldolase 2
Lys	13.8	22.7
His	6.0	10.2
Arg	12.5	7.6
CmCys	2.2	0.86
Asp	39.5	33.5
Thr*	17.4	17.6
Ser*	20.8	25.6
Glu	28.2	35.3
Pro	10.7	12.5
Gly	27.7	30.4
Ala	37.6	29.1
Val†	20.8	22.6
Met	6.4	6.0
Ile†	17.3	16.4
Leu	30.6	24.4
Tyr	16.3	11.5
Phe	9.7	11.5

* Corrected for destruction during 24h hydrolysis.
† Incompletely released during 24h hydrolysis.

in conjunction with the estimated subunit molecular weight, suggests that aldolase 1 is a tetramer with total mol.wt. about 140000 (eluted immediately after rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, mol.wt. 144000). Aldolase 2, which has the same subunit molecular weight as aldolase 1, is, however, clearly separable on gel-filtration columns. Its elution position just preceding bovine serum albumin (mol.wt. 68000) suggests a dimeric form of mol.wt. 70000 in 50mM-Tris–HCl buffer, pH7.5.

Amino acid analyses. The amino acid compositions of aldolase 1 and aldolase 2 are shown in Table 1.

Treatment of aldolases 1 and 2 with EDTA. Solutions of EDTA of various concentrations, adjusted to pH7.5 with NaOH, were added to the colorimetric assay mixture from which substrate was omitted. A fixed amount of either aldolase 1 or aldolase 2 was added and the mixture was incubated at room temperature for 10min. The assay was then started by adding 0.05ml of 50mM-fructose diphosphate. Aldolase 1 activity was not inhibited even by high concentrations of EDTA whereas aldolase 2 activity was 50%
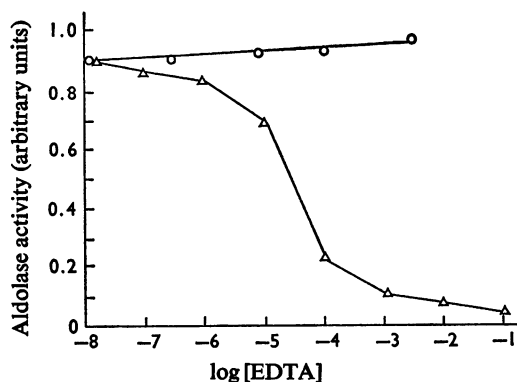
Fig. 2. *Effect of EDTA on aldolase 1 and aldolase 2 activities*

EDTA, buffered at pH7.5 with 50mM-Tris–HCl, was added to the colorimetric assay mixture before addition of substrate. Standard amounts (20µg) of either aldolase 1 or 2 were then added and the mixture was incubated for 10min at 20°C before the addition of substrate. The concentrations of EDTA (M) shown are the final values in the assay mixture without substrate. ○, Activity of aldolase 1; △, activity of aldolase 2 (arbitrary units).

Table 2. *Effect of bivalent metal ions on the activity of apoenzyme and holoenzyme forms of aldolase 2*

Metal ions or EDTA were added to the colorimetric assay mixture and incubated for 10min with a fixed amount of enzyme (20µg) before the addition of substrate. The activities of the holoenzyme and of the apoenzyme preparation are expressed as % of activity with no additions in each case.

Additions	Holoenzyme activity (%)	Apoenzyme activity (%)
None	100	100
1mM-Ca²⁺	92	98
1mM-Co²⁺	74	237
1mM-Zn²⁺	72	131
1mM-EDTA	16	16

inhibited by 30µM-EDTA (see Fig. 2).

Activation of aldolase 2 by bivalent metal ions. Aldolase 1 was not activated by any of the bivalent metal ions tested (Cu^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Ca^{2+}, Fe^{2+} etc.) and in some cases showed a marked inhibition. Aldolase 2, when present solely as the holoenzyme, showed a similar response to metal ion additions (Table 2). A mixture of apoenzyme and holoenzyme was prepared by incubating aldolase 2

(5 mg/ml) with 1 mM-EDTA for 2 h in 50 mM-Tris–HCl buffer, pH 7.5. The EDTA was removed by gel filtration on a small column (15 cm × 1 cm) of Sephadex G-25 equilibrated with 50 mM-Tris–HCl buffer, pH 7.5, that had been extracted with dithizone (diphenylthiocarbazone; 1 mg/ml in chloroform) to remove contaminating metal ions. The apoenzyme–holoenzyme mixture thus obtained was activated by Zn^{2+}, Co^{2+}, and Fe^{2+} ions added to the colorimetric assay mixture. The behaviour of aldolase 2 with different additions is illustrated in Table 2. Co^{2+} and Zn^{2+}, which inhibit the holoenzyme, activate the apoenzyme but the relative ability of the two metal ions to activate varies with pH. Ca^{2+} (1 mM) was added in one experiment to show that the effect of added metal ions is not merely to remove EDTA from a firmly bound metal ion in the active site. A second addition of 1 mM-EDTA to the mixture of holoenzyme and apoenzyme showed that the residual activity was still sensitive to EDTA. The ion that is bound to the apoenzyme *in vivo* has not been determined.

Effect of NaBH₄ on aldolases 1 and 2. Solutions of aldolase 1 and aldolase 2 (2 mg/ml) were maintained at pH 6.0 in a pH-stat with 2 M-acetic acid as titrant during the addition of 1 M-NaBH₄ to a final concentration of 100 mM. The effect of borohydride was investigated in the absence and in the presence of fructose diphosphate and dihydroxyacetone phosphate. Samples of enzyme were tested for activity by using the coupled assay.

Aldolase 1 activity was completely inhibited by borohydride in the presence of either 10 mM-fructose diphosphate or 7 mM-dihydroxyacetone phosphate within 5 min of the first addition of borohydride. When neither substrate was present, borohydride caused a 30% inactivation at the end of 1 h. This inactivation was decreased by controlling the foaming with a drop of octan-2-ol, and slowing the rate of addition of borohydride. Aldolase 2 was not rapidly affected by borohydride in the presence of either substrate, but showed a slight and variable inactivation, independent of substrate, after 1 h.

A more controlled reduction was attempted by using NaBCNH₃ at pH 7.0. Cyanoborohydride is reported to be more specific for the reduction of imines at pH 6–8 and is stable down to pH 3.0 (Borch *et al.*, 1971). Although only a very slow inactivation of aldolase 1 was observed with 1 mM-cyanoborohydride and 10 mM-fructose diphosphate, the enzyme was 85% inactivated after 3 h if the concentration of cyanoborohydride was raised to 100 mM. In the absence of substrate there was no inactivation. However, aldolase 1 incubated with 7 mM-dihydroxyacetone phosphate and 100 mM-cyanoborohydride showed only 5% inhibition after 3 h. This apparent anomaly may be explained since the dihydroxyacetone phosphate is probably being used at concen-

trations near its K_m value whereas the enzyme will be fully saturated at 10 mM-fructose diphosphate (see below). In addition, the concentration of available
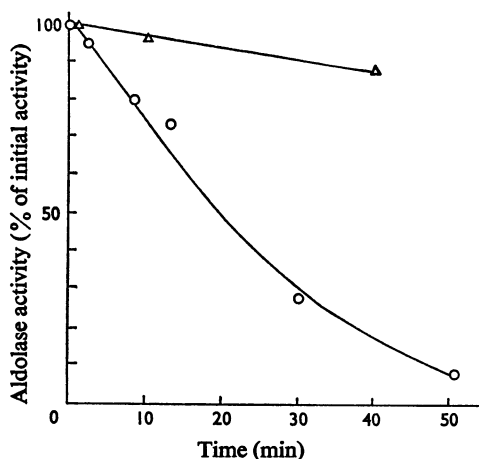

Fig. 3. *Effect of N-ethylmaleimide on the activity of aldolases 1 and 2*

Aldolase 1 and aldolase 2 were incubated at 15°C with 0.5 mM-*N*-ethylmaleimide at pH 7.5. Samples were tested for activity in the coupled assay and the results are expressed in terms of the initial enzymic activity as 100% in each case. ○, Activity of aldolase 1; △, activity of aldolase 2.
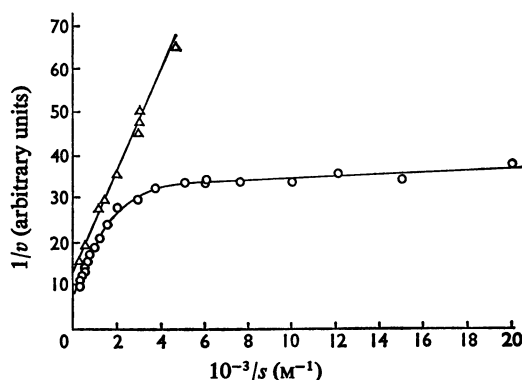

Fig. 4. *Lineweaver–Burk plot for aldolase 1 and aldolase 2, with fructose diphosphate*

The activities of fixed amounts of aldolases 1 and 2 were measured at pH 7.5 and 30°C in the coupled assay by using different concentrations of fructose diphosphate. The absolute concentration of fructose diphosphate in the stock solution was determined enzymically by using rabbit muscle aldolase in the same system. ○, Aldolase 1; △, aldolase 2.

dihydroxyacetone phosphate may be further decreased by any free cyanide ions released from the cyanoborohydride.

Reaction with N-ethylmaleimide. Aldolase 1 and aldolase 2 (1 mg/ml) were treated with N-ethylmaleimide (0.5 mM) at 15°C in 50 mM-Tris–HCl buffer, pH 7.5. Samples taken at intervals were tested in the coupled assay, which was not affected by the small concentration of N-ethylmaleimide added with the enzyme sample. Aldolase 1 showed 50% inhibition after 20 min, whereas aldolase 2 showed only 35% inhibition after 4 h (see Fig. 3).

Kinetic behaviour of aldolase 1 and aldolase 2. The kinetics of the two enzymes for the aldol cleavage reaction were studied by using the coupled assay. Aldolase 1 with fructose diphosphate gave a non-linear Lineweaver–Burk plot (see Fig. 4) but with fructose 1-phosphate the plot was linear (Fig. 5). It is difficult to assess the true K_m value for aldolase 1 with fructose diphosphate, but an apparent K_m value of 20 μM may be calculated from the gradient of the linear section of the plot. The apparent activation by

high substrate concentrations was not due to Na^+ ions added with the fructose diphosphate, since equivalent concentrations of NaCl did not cause the activation. The relevance *in vivo* of the activation by high substrate concentrations is not clear. It is conceivable that some proteolysis may have occurred during the purification of aldolase 1 that has produced a mixture of native enzyme and a partially digested form, with altered affinity for substrate and decreased specific activity. This would lead in turn to a non-linear Lineweaver–Burk plot. However, the digestion would have to be restricted to a small number of residues at one or both ends of the polypeptide chain to avoid the appearance of two bands on sodium dodecyl sulphate–polyacrylamide gels. Inclusion of EDTA (1 mg/ml) throughout the purification of aldolase 1 did not affect the kinetic properties of the final crystalline product, which makes it unlikely that the proteolysis (if it has occurred) is dependent on metal ions.

Aldolase 2 gave a linear Lineweaver–Burk plot with fructose diphosphate (Fig. 4), but the K_m value for the enzyme with fructose 1-phosphate is so high that its accurate measurement is impossible. The kinetic constants for the two enzymes are summarized in Table 3.

Discussion

Classification of aldolase 1 and aldolase 2 is possible on the basis of experiments described in the present paper. Aldolase 1 has many of the properties expected of a class I aldolase, e.g. the irreversible inhibition by borohydride in the presence of substrate, the tetrameric form of the enzyme, the sensitivity to N-ethylmaleimide (Anderson & Perham, 1970) and the K_m value for fructose 1,6-diphosphate and for fructose 1-phosphate. On the other hand, aldolase 2 is strongly inhibited by EDTA and is reactivated by bivalent metal ions. The K_m value for fructose diphosphate and the dimeric form of the enzyme (mol.wt. 70000) are typical of a class II aldolase. In these respects aldolase 2 corresponds to the aldolase of *E. coli* described by Doelle & Manderson (1971).

There is good circumstantial evidence, therefore, that aldolases 1 and 2 belong to classes I and II respectively. If amino acid sequence analysis con-
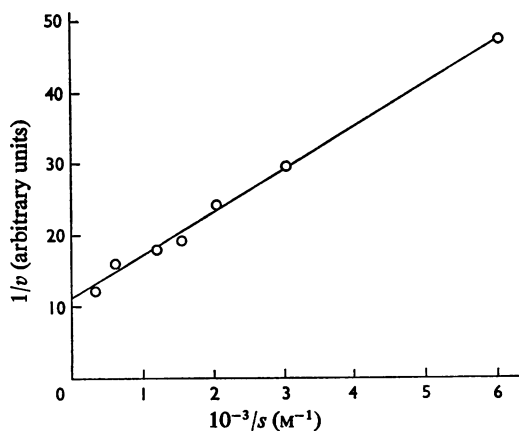

Fig. 5. *Lineweaver–Burk plot of aldolase 1 with fructose 1-phosphate*

The activity of a fixed amount of aldolase 1 was determined in the coupled assay at 30°C and pH 7.5, with different concentrations of fructose 1-phosphate.

Table 3. *Kinetic constants for aldolases 1 and 2 with fructose 1-phosphate and fructose diphosphate*

The activities of aldolase 1 and aldolase 2 were determined in the presence of various concentrations of fructose 1-phosphate and fructose diphosphate, by using the coupled assay system. The values of K_m and V were calculated from Lineweaver–Burk plots (Figs. 4 and 5).

Enzyme	K_m (M) (fructose 1-phosphate)	K_m (M) (fructose diphosphate)	$\dfrac{V_{\text{fructose diphosphate}}}{V_{\text{fructose 1-phosphate}}}$
Aldolase 1	4.5×10^{-4}	2×10^{-5}	5
Aldolase 2	Approx. 0.1	8.5×10^{-4}	20

firms these assignments, this will be the first tetra-meric fructose diphosphate aldolase that involves imine formation with substrate to be identified in bacteria. It should be noted, however, that unusual bacterial aldolases that operate via a Schiff-base mechanism have already been reported, e.g. 2-deoxy-D-ribose 5-phosphate aldolase from *Lactobacillus plantarum* (Grazi *et al.*, 1963), 3-deoxy-2-oxo-6-phosphogluconate aldolase from *Pseudomonas fluor-escens* (Grazi *et al.*, 1963; Ingram & Wood, 1966) and *N*-acetylneuraminic acid aldolase from *Clostridium perfringens* (De Vries & Binkley, 1972). Class I and class II aldolases have also been described in the green algae *Chlamydomonas* and *Euglena* (Rutter, 1964; Russell & Gibbs, 1967; Guerrini *et al.*, 1971). It is noteworthy that in these photosynthetic organisms the presence and amount of the two enzymes depends on the growth conditions. During growth with CO_2 as carbon source, only class I aldolase is produced, but when acetate replaces CO_2 the class II aldolase is made (Rutter, 1965; Russell & Gibbs, 1967; Guerrini *et al.*, 1971). Similarly, in *E. coli* the putative class I aldolase is only synthesized during growth on C_3 compounds (pyruvate and lactate) and not on glucose, implying that a class I aldolase is preferred for gluconeogenesis. A similar situation exists in mammalian muscle (glycolytic) and liver (gluconeogenic), where class I aldolases with appro-priately different kinetic properties are found in the two tissues (see Morse & Horecker, 1968).

The two types of aldolase available from *E. coli* grown on lactate or pyruvate should now provide an ideal system for a study of the genetic and functional relations between class I and class II aldolases, since there is no complication of species diversity. It is worth noting that the two aldolases in *E. coli* have an identical subunit molecular weight, typical of class II aldolases but significantly smaller than that common-ly associated with the aldolases of class I. On the other hand, there is no obvious similarity between the amino acid compositions (Table 1), and any common genetic ancestry will only be uncovered by more detailed analysis of amino acid sequences. The class I aldolases of mammalian muscle and liver, although homo-logous, are characteristically different (Forcina & Perham, 1971). In view of the fact that the synthesis of the putative class I aldolase of *E. coli* is favoured by conditions of gluconeogenesis, it will be of particular interest to unravel the evolution of these three en-zymes and their relation to the class I aldolases of plants, e.g. spinach (Ribereau-Gayon *et al.*, 1971).

Note added in proof (*received* 19 *January* 1973)

A monomeric fructose diphosphate aldolase from *Micrococcus aerogenes* that uses a Schiff-base mech-anism has recently been discovered by H. G. Lebherz,

R. A. Bradshaw & W. J. Rutter (personal communi-cation and unpublished work quoted by Lebherz 1972).

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