Biochem. J. (1972) **129**, 301–310
Printed in Great Britain

Specificity and Kinetics of Triose Phosphate Isomerase from Chicken Muscle

By SYLVIA J. PUTMAN, A. F. W. COULSON, I. R. T. FARLEY, B. RIDDLESTON and J. R. KNOWLES
The Dyson Perrins Laboratory, University of Oxford, Oxford OX1 3QY, U.K.

(*Received* 16 *March* 1972)

The isolation of crystalline triose phosphate isomerase from chicken breast muscle is described. The values of $k_{cat.}$ and K_m for the reaction in each direction were determined from experiments over wide substrate-concentration ranges, and the reactions were shown to obey simple Michaelis–Menten kinetics. With D-glyceraldehyde 3-phosphate as substrate, $k_{cat.}$ is $2.56 \times 10^5 min^{-1}$ and K_m is 0.47 mM; with dihydroxyacetone phosphate as substrate, $k_{cat.}$ is $2.59 \times 10^4 min^{-1}$ and K_m is 0.97 mM. The enzyme-catalysed exchange of the methyl hydrogen atoms of the 'virtual substrate' monohydroxyacetone phosphate with solvent ^2H_2O or ^3H_2O was shown. This exchange is about 10^4-fold slower than the corresponding exchange of the C-3 hydrogen of dihydroxyacetone phosphate. The other deoxy substrate, 3-hydroxypropionaldehyde phosphate, was synthesized, but is too unstable in aqueous solution for analogous proton-exchange reactions to be studied.

Triose phosphate isomerase (D-glyceraldehyde 3-phosphate ketol-isomerase; EC 5.3.1.1) catalyses the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. The enzyme has been isolated from a variety of sources, including calf muscle (Meyer-Arendt *et al.*, 1953), rabbit muscle (Czok & Bücher, 1960; Norton *et al.*, 1970), horse and human liver (Lee *et al.*, 1971), yeast (Krietsch *et al.*, 1970*a*), bovine lens (Burton & Waley, 1968*a*), pea seedlings (Turner *et al.*, 1965), algae (Meeks *et al.*, 1968), and a thermophilic bacterium (Fahey *et al.*, 1971). Primarily because the enzyme in chicken muscle extract shows only one major band on starch-gel electrophoresis, whereas that from a number of other sources has several bands (Scopes, 1968), the chicken muscle enzyme was prepared (Trentham *et al.*, 1969) and found to crystallize appropriately for X-ray crystallographic work (Johnson & Wolfenden, 1970; Banner *et al.*, 1971).

In order that the kinetic and mechanistic work described in the present and the following papers (Plaut & Knowles, 1972; de la Mare *et al.*, 1972) should complement the crystallographic studies, a simple preparation was devised for triose phosphate isomerase from chicken breast muscle. We describe in the present paper the characteristics of the enzyme-catalysed reaction in each direction. The isolation procedure is only reported in outline, since the method has been further simplified by McVittie *et al.* (1972). Experiments with the two des-hydroxy substrate analogues are reported, as well as studies of the catalysed reaction rate in each direction over wide concentration ranges.

Experimental

Materials

Chicken muscle. Commercial frozen chickens for domestic consumption, of less than 2kg, were purchased from MacFisheries Ltd., Oxford, U.K. After thawing overnight in the cold room, the breast muscle was removed for processing.

DEAE-cellulose. DE 52 DEAE-cellulose, was purchased from W. and R. Balston Ltd., Maidstone, Kent, U.K.

Polyethylene glycol. This, as 'Carbowax 6000', was purchased from Union Carbide (U.K.) Ltd., London W.1.

$(NH_4)_2SO_4$. This was AnalaR grade, from British Drug Houses Ltd., Poole, Dorset, U.K. For crystallization purposes, the higher grade ('specially low in heavy metals for enzyme work') was used.

Acetonylacetate. This was obtained from Ralph Emanuel Ltd., Wembley, Middx., U.K.

Assay components. These were as described by Plaut & Knowles (1972).

Aldolase. Rabbit muscle aldolase was purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Crystalline yeast aldolase was a kind gift from R. Jack and J. I. Harris, Laboratory of Molecular Biology, Cambridge, U.K.

Enzymes. Porcine pepsin (twice crystallized), bovine serum albumin (crystallized) and equine myoglobin (crystallized) were obtained from Sigma (London) Chemical Co. Ltd.

Buffer components and other chemicals. These were

of the highest grade available. Deionized water was used for all solutions.

Monohydroxyacetone phosphate. This was prepared as the dicyclohexylammonium salt of the diethyl ketal by a modification of the method of Sellinger & Miller (1958). The diethyl ketal of acetonylacetate was hydrolysed at room temperature in the pH-stat, 1M-NaOH being used to maintain the pH at 11.0. The product, monohydroxyacetone diethyl ketal, was isolated by extraction into diethyl ether, and purified by fractional distillation. This procedure gives better yields than the calcium oxide method used by Sellinger & Miller (1958). Phosphorylation with diphenyl phosphochloridate followed by deprotection of the phosphate ester gave monohydroxyacetone phosphate diethyl ketal as its dicyclohexylammonium salt. It had m.p. 149–151°C (decomp.) [Sellinger & Miller (1958) give 147–149°C (decomp.)], but gave an unsatisfactory micro-analysis (Found: C, 47.6; H, 10.0; N, 5.8; P, 6.9; $C_{19}H_{43}O_6N_2P$ requires C, 51.4; H, 10.2; N, 6.3; P, 7.1%). This is commonly found with dicyclohexylammonium salts and has been attributed to the loss of cyclohexylamine during drying (McGilvery, 1953). The n.m.r. spectrum (at 60MHz) in ^2H_2O showed peaks at 5.5τ (doublet of $J = 6$Hz, two protons, $-CO-CH_2-OPO_3{}^2H_2$), and a broad resonance between 7.5 and 9.0τ (multiplet due to cyclohexylamine protons) superimposed on which was a triplet at 8.7τ (CH_3-CH_2-O-).

The diethyl ketal salt was converted into free monohydroxyacetone phosphate as described by Sellinger & Miller (1958). The salt (250mg) was dissolved in water (1ml) and the solution added to a suspension of Dowex 50 (H^+ form) ion-exchange resin (1.8g) in water (5ml). After swirling, the mixture was made up to 25ml with water, and incubated at 40°C for 3h. The resin was then removed by filtration, and the concentration of monohydroxyacetone phosphate determined (*a*) by assaying for P_i before and after hydrolysis, and (*b*) by estimation of the total ketone present (see below). Phosphate was assayed by the method of Briggs (1922), with AnalaR NaH_2PO_4 to calibrate the method. This test responds only to P_i. Before hydrolysis, phosphate assay of the solution of monohydroxyacetone phosphate gave 0.9mM. After complete hydrolysis of the ester [by heating monohydroxyacetone phosphate solution (1ml) with 10M-NaOH (4ml) at 80°C for 10h], the P_i concentration was 28.0mM. The original ester solution is therefore contaminated with 3.5% of free phosphate. The concentration of monohydroxyacetone phosphate was checked by assaying for free carbonyl groups by the method of Lappin & Clark (1951). Acetonylacetate was used to calibrate the method. A concentration of 28.75mM was obtained, in good agreement with the phosphate ester analysis.

Solutions of monohydroxyacetone phosphate for n.m.r. work were prepared by dissolution of the freeze-dried free phosphate ester in ^2H_2O.

Randomly ^3H-labelled monohydroxyacetone phosphate for use in enzyme-catalysed detritiation experiments was prepared as follows. A solution of monohydroxyacetone phosphate (approx. 10mM) was made 0.1M with respect to NaOH and ^3H_2O was added to a final concentration of 1.25mCi/ml. After incubation at 37°C for 12h, the solution was neutralized and chromatographed on a column (4cm × 1cm) of Dowex 1 (Cl^- form). Excess of radioactivity (^3H_2O, and any [^{3}H]hydroxyacetone resulting from base-catalysed phosphate ester hydrolysis) was washed out with 0.001M-HCl, and the labelled ester was eluted with 0.03M-HCl.

3-Hydroxypropionaldehyde phosphate. This was prepared by phosphorylating the diethyl acetal of 3-hydroxypropionaldehyde.

Hydracrylaldehyde acetate was prepared by the method outlined by Fischer & Smith (1959). A glass column was charged with Amberlite IRA-400 anion-exchange resin, and was soaked overnight with 2.5M-NaOH solution. After the column had been washed with water until the eluate was neutral, it was equilibrated with acetic acid. Acrolein (97%, stabilized with quinol) (97.5 parts) was mixed with a 1:1 (v/v) mixture of acetic acid and water (2.5 parts), and the solution was applied to the column. After 24h at room temperature, the solution was drained from the column and subjected to fractional distillation. The hydracrylaldehyde acetate [b.p. 60–62°C/133N/m^2 (1mmHg)] was obtained in 30% yield, and had n_D^{18} 1.4183 [Ballard *et al.* (1951) give n_D^{20} 1.4179]. The 2,4-dinitrophenylhydrazone had m.p. 123–124°C [Ballard *et al.* (1951) give 124.5–125.5°C].

The diethyl acetal was prepared as follows. A mixture containing hydracrylaldehyde acetate (23g), triethyl orthoformate (30g), ethanol (2ml) and conc. H_2SO_4 (2 drops), was kept at room temperature for 48h. The resulting brown solution was neutralized with 2M-NaOH and was fractionally distilled. The diethyl acetal [b.p. 76–79°C/133N/m^2(1mmHg)] was obtained in 37% yield (Found: C, 57.0; H, 9.5; $C_9H_{18}O_4$ requires C, 56.8; H, 9.5%). The n.m.r. spectrum (at 100MHz) in C^2HCl_3 solution showed peaks at 5.3τ [triplet, 1 proton, $-CH_2-CH(O-CH_2-CH_3)_2$], 5.75τ (triplet, 2 protons, $CH_3-CO_2-CH_2-CH_2-$), 6.30τ (multiplet, 4 protons, CH_3-CH_2-O-), 7.85τ (singlet, 3 protons, CH_3-CO_2-), 7.95τ [quartet, 2 protons, $-CH_2-CH_2-CH(O-CH_2-CH_3)_2$], and 8.7$\tau$ (triplet, 6 protons, CH_3-CH_2-O-).

Hydrolysis of the acetate was done in the pH-stat at pH10.5 and room temperature. The product, 3-hydroxypropionaldehyde diethyl acetal, was extracted into ether and purified by fractional distillation. The yield was 69% [b.p. 82–86°C/665N/m^2 (5mmHg)]. The n.m.r. spectrum (at 60MHz) in C^2HCl_3 solution showed peaks essentially identical

with those of the acetate (above) except that the peak at 7.85τ (CH_3–CO_2–) was absent.

Phosphorylation of 3-hydroxypropionaldehyde diethyl acetal (4.5g) in dry pyridine (20ml) was done by the slow addition of diphenyl phosphochloridate (11g) (Baer, 1952) to the ice-cold acetal solution. The mixture was left overnight at 4°C. The precipitate of pyridine hydrochloride was removed by filtration and a few drops of water added to destroy excess of diphenyl phosphochloridate. The solvent was removed under reduced pressure, and the resulting oil was dissolved in chloroform and washed successively with water, 0.1M-HCl, 0.1M-Na_2CO_3, and water. After drying the solution over anhydrous Na_2SO_4, the solvent was evaporated, and the product was purified by chromatography on a column of silica gel with benzene–ether (19:1, v/v) as eluent. The yield of product, diphenylphosphorylhydroxypropionaldehyde diethyl acetal, was 79% (Found: C, 60.5; H, 6.4; P, 8.5; $C_{19}H_{25}O_6P$ requires C, 60.0; H, 6.6; P, 8.2%).

This diphenyl phosphate ester (4g) was hydrogenolysed in dry ethanol (60ml) in the presence of platinum oxide (0.25g) at atmospheric pressure until the theoretical volume of H_2 had been adsorbed (approx. 3h). The catalyst was removed by filtration, and water (50ml) was added to the filtrate. The pH of this solution was adjusted to about pH9.5 with aqueous 1M-cyclohexylamine. The solvent was then removed under reduced pressure and the product was obtained as an off-white solid. Recrystallization from $CHCl_3$–light petroleum (b.p. 40–60°C) yielded the dicyclohexylamine salt of 3-hydroxypropionaldehyde phosphate as a white solid in 54% yield, with m.p. 150–152°C (decomp.) (Found: C, 51.3; H, 10.1; N, 6.2; P, 7.3; $C_{19}H_{43}O_6N_2P$ requires C, 51.4; H, 10.2; N, 6.3; P, 7.1%). The n.m.r. spectrum (at 60MHz) in ^2H_2O solution gave a complex signal centred at 6.25τ, approximating to two overlapping triplets (–CH_2–CH_2–$OPO_3{}^2H_2$), and a broad resonance between 7.5–9.0τ (multiplet due to cyclohexylamine protons) superimposed on which was a triplet centred at 8.8τ (CH_3–CH_2–O–).

The diethyl acetal was converted into free 3-hydroxypropionaldehyde phosphate by the method described above for the liberation of monohydroxyacetone phosphate. In this case the precursor was incubated with the resin for 4h at 40°C. Phosphate analysis of the resulting solution showed significant contamination (11% of total phosphate) by P_i, which presumably arises from the relatively facile hydrolysis of this phosphate ester (see the Results section).

Sodium tetrathionate. This was prepared as described by Gilman *et al.* (1946).

^2H_2O. This was obtained from Norsk Hydroelektrisk (through Rivan Chemicals, West End, Southampton, U.K.), and was of 99.7% isotopic purity.

^3H_2O. This (5Ci/ml) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Enzyme assays. These were performed at 30°C under the conditions described by Plaut & Knowles (1972). In the present work, a wider range of substrate concentrations was studied, and the validity of the assays was checked by ensuring the linear dependence of the observed initial rate on the concentration of triose phosphate isomerase under the highest substrate concentration conditions used. In calculations involving enzyme active site concentration a subunit molecular weight of 25000 is assumed and the concentration is expressed as a normality rather than a molarity.

Scintillation counting. This was done with an automatic Beckman DPM-100 instrument, by using a scintillation fluid made up with 2,5-diphenyloxazole (7.0g), 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.24g), Grignard-dry ethanol (640ml) and AnalaR toluene (1400ml). Portions (3 or 4ml) of this solution were used with 50μl or 100μl aqueous portions of sample. Counting efficiency was approx. 30%.

N.m.r. spectra. These were obtained by Mrs. E. Richards in this Department, with either a Perkin–Elmer R.10 or R.14 spectrometer.

pH measurements. These were made with a Radiometer TT1c instrument with a pHA 630 scale-expander attachment, standardized against standard buffer solutions from British Drug Houses Ltd. pH-stat runs were performed with the Radiometer Titrator TTT1c and Titrigraph SBR 2c.

Polyacrylamide gel electrophoresis. This was done as described by Davis (1964) and Shapiro *et al.* (1967).

Isolation of triose phosphate isomerase. Chicken breast muscle was minced in a power mincer (Hobart Mfg. Co. Ltd., Bedford, U.K.) at 4°C, and gently stirred with its own weight of 1.3mM-EDTA (disodium salt), pH7.5, 1mM in 2-mercaptoethanol, for 30min. The extract was centrifuged at 650*g* and 4°C for 30min. The residue was re-extracted with EDTA solution (0.5ml/g of original muscle), and the combined supernatants were filtered through cheesecloth. Finely ground $(NH_4)_2SO_4$ was added slowly, with stirring, to a concentration of 2.6M and the solution was left overnight. After removal of the precipitate by centrifugation at 8500*g* and 4°C for 40min, the supernatant was filtered and then dialysed at 4°C for 4h against 10mM-triethanolamine–HCl buffer, pH7.7, containing 1mM-EDTA. The dialysis bags were then transferred to the same buffer containing 30% (w/v) polyethylene glycol, until the total protein concentration was around 35mg/ml. This concentrate was dialysed exhaustively against the same buffer (containing no polyethylene glycol) and then was subjected to equilibrium chromatography

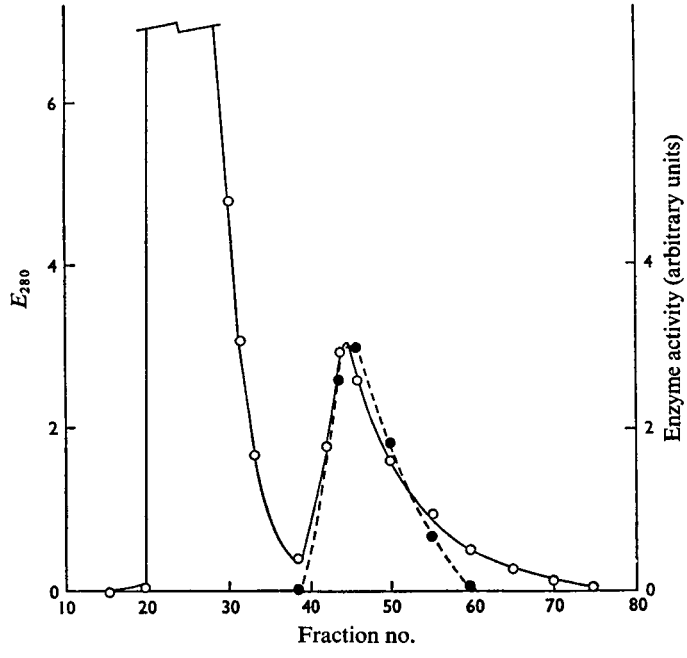


Fig. 1. *Elution profile of crude triose phosphate isomerase from DEAE-cellulose*

The sample (100ml), in 10mM-triethanolamine–HCl buffer, pH7.7, containing 1mM-EDTA, was applied to the column (5cm×40cm) of DEAE-cellulose and was eluted with the same buffer. The fraction size was 14ml. o, E_{280}; ●, enzyme activity.

Table 1. *Purification of triose phosphate isomerase from chicken muscle*

The enzyme preparation was from 470g of chicken breast muscle. Protein was measured by using an $E_{280}^{0.1\%}$ appropriate to isomerase of 1.21. Activity is given in units and one unit is defined as 1 μmol of D-glyceraldehyde 3-phosphate consumed/min under substrate saturation conditions, in 50mM-triethanolamine–HCl buffer, pH7.5, at 30°C.

	Total protein (mg)	$10^{-3}\times$Total activity (units)	Specific activity (units/mg)	Yield (%)
Extract	21400	5940	278	100
$(NH_4)_2SO_4$ fraction	3390	4100	1210	69
DEAE-cellulose fraction	570	3320	5820	56
Crystals (2×crystallized)	216	2420	11200	41

at room temperature on DEAE-cellulose (1.5ml/g of original muscle) in 10mM-triethanolamine–HCl buffer, pH7.7, containing 1mM-EDTA. The elution profile is shown in Fig. 1. Fractions containing triose phosphate isomerase of greater than 70% purity (assuming a specific activity for the purified enzyme of 11000 units/mg) were pooled, and concentrated by dialysis at 4°C against the above triethanolamine–HCl buffer containing polyethylene glycol until the protein concentration was about 10mg/ml. Solid $(NH_4)_2SO_4$ (especially low in heavy metals) was added until a slight turbidity appeared. Hexagonal bipyramidal crystals appeared within a few hours. The enzyme was recrystallized twice. This material ran as a single band on polyacrylamide-gel electrophoresis at pH9.3, and had a specific activity of 11000 units/mg at 30°C (see Table 1). The isolation of chicken muscle triose phosphate isomerase by a

different method has been reported in outline by Trentham *et al.* (1969).

Titration of thiol groups. All measurements were made at room temperature using a double-beam u.v. spectrophotometer. Each cuvette contained 2ml of 3.5mM-5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959) in 0.5M-potassium phosphate buffer, pH7.6, 10mM with respect to EDTA. Enzyme solution (400μl of approx. 0.1mN solution in 50mM-tris–HCl buffer, pH8.0) was added to the sample cuvette and a portion (400μl) of this tris–HCl buffer was added to the reference cuvette. The absorbance at 412nm was monitored as a function of time, the molar extinction coefficient of Ellman reagent anion being taken as 13600 (Ellman, 1959). Under these conditions, reaction of 'accessible' thiol groups was complete within 30min. 'Total' thiol groups were estimated as above, with sodium dodecyl sulphate being added to 0.5% (w/v) either before the addition of enzyme, or after the complete reaction of 'accessible' thiol groups. Reaction of 'total' thiol groups was complete within a few seconds.

Results and Discussion

Properties of chicken muscle triose phosphate isomerase

The enzyme isolated as described above migrates as a single band on polyacrylamide-gel electrophoresis, in the presence and absence of sodium dodecyl sulphate. The molecular heterogeneity of crystalline isomerase from rabbit muscle (see Norton *et al.*, 1970; Krietsch *et al.*, 1970*b*) is evidently not apparent with enzyme from chicken breast muscle. This agrees with the distribution of isomerase activity on starch-gel electrophoresis of muscle extracts from different species reported by Scopes (1968).

The subunit molecular weight of triose phosphate isomerase as determined by gel electrophoresis in the presence of sodium dodecyl sulphate (Shapiro *et al.*, 1967) is 26500 (see Fig. 2). This is in good agreement with the value of 26200 obtained for the asymmetric unit of rabbit muscle isomerase crystals, which show a twofold rotation axis relating the two subunits (Johnson & Waley, 1967). Equilibrium ultracentrifugation of the chicken muscle enzyme gives 48400 (M. P. Esnouf, personal communication), and of the rabbit muscle enzyme gives 52900 (Norton *et al.*, 1970).

The enzyme from chicken muscle has one thiol group per subunit that is more reactive than the others. Titration with Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid); Ellman, 1959] results in the essentially complete reaction of one group per subunit; the remaining three thiol groups can only react after the protein has been unfolded in 0.5% (w/v) sodium dodecyl sulphate (Table 2). Modification of this single thiol group can be effected by sodium tetrathionate (Pihl & Lange, 1962) with little loss in the catalytic activity. Titration with Ellman's reagent shows that the 'fast-reacting' thiol group has been modified. Re-liberation of this thiol group by incubation of the modified enzyme with dithiothreitol is quantitative, with retention of the catalytic activity (Table 2). It appears therefore that each subunit of isomerase contains one accessible and three inaccessible thiol groups, the accessible group being inessential to the catalytic activity of the enzyme. This contrasts with the rabbit muscle enzyme, for which Krietsch *et al.* (1971) have suggested that one thiol group per dimer can be modified with Ellman's reagent with the loss of 50% of the enzyme activity, and that 'a second essential SH group corresponding to the other active site, still fractionally intact, is inaccessible ... through the association of the two subunits.'

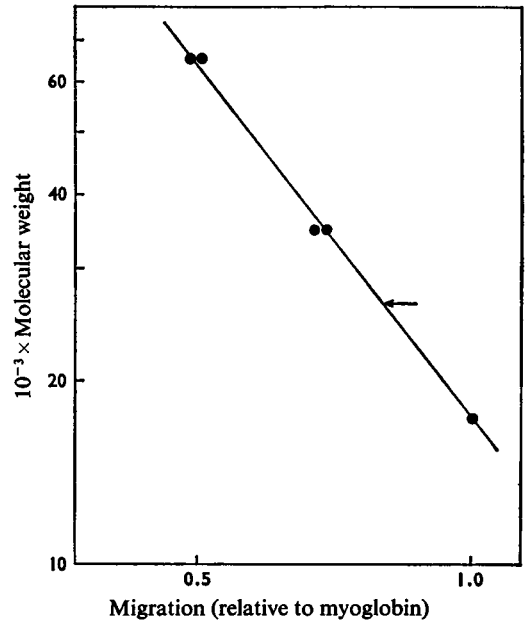


Fig. 2. *Semilogarithmic plot of subunit molecular weight versus migration in polyacrylamide gels in sodium dodecyl sulphate*

For experimental details see the text. The following standards were used: bovine serum albumin (mol.wt. 65500); pepsin (mol.wt. 34000); and myoglobin (mol.wt. 17200). The position of triose phosphate isomerase is shown by the arrow.

Table 2. *Modification of thiol groups in chicken muscle triose phosphate isomerase*

'Accessible' thiol groups are those reacting with Ellman's reagent (Ellman, 1959) in the absence of sodium dodecyl sulphate. 'Total' thiol groups are those reacting with Ellman's reagent in the presence of 0.5% (w/v) sodium dodecyl sulphate. For details, see the Experimental section.

Enzyme	Catalytic activity (% of native)*	'Accessible' thiol groups/subunit	'Total' thiol groups/subunit
Native	100	1.0	3.7
Enzyme treated with tetrathionate†	85	0.0	2.8
Control‡	100	1.0	3.6
Enzyme treated with tetrathionate and then with dithiothreitol§	91	1.0	3.3
Control‖	88	1.0	3.4

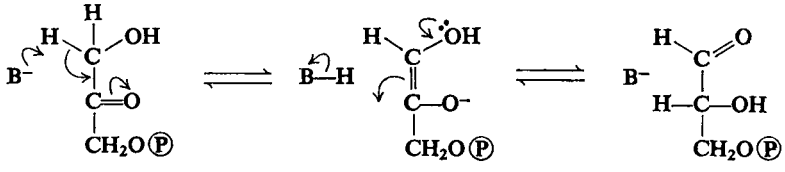
* Catalytic activity was measured on protein samples that had been exhaustively dialysed against 50mM-tris–HCl buffer, pH8.0.

† Enzyme (approx. 0.2μN) was treated with a 200-fold molar excess of fresh sodium tetrathionate for 1h, at room temperature, in 0.1M-tris–HCl buffer, pH8.0. The enzyme was then dialysed exhaustively against 50mM-tris–HCl buffer, pH8.0.

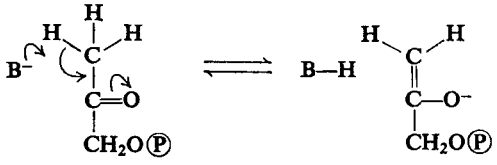
‡ This control sample was subjected to the same sequence of incubation and dialysis as the tetrathionate-treated material, except for the absence of sodium tetrathionate.

§ The tetrathionate-treated enzyme was incubated with 10mM-dithiothreitol for 16h, at 0°C, in 0.1M-tris–HCl buffer, pH8.6. The enzyme was then dialysed exhaustively against 50mM-tris–HCl buffer, pH8.0.

‖ This control sample was subjected to the same sequence of incubation with dithiothreitol and dialysis as the tetrathionate-treated material, except that native enzyme (unmodified by sodium tetrathionate) was used.



Eqn. (1)



Eqn. (2)

Scheme 1. *Interaction of substrates and of a 'virtual substrate', with triose phosphate isomerase*

Eqn. (1) shows reaction of substrate with isomerization and eqn. (2) shows reaction of 'virtual substrate' with no isomerization.

Specificity of triose phosphate isomerase

Although a considerable number of anionic materials are competitive inhibitors of triose phosphate isomerase (for a partial list, see Wolfenden, 1969), it was important both for mechanistic and for structural studies to find if either of the deoxy substrate analogues (i.e. 3-hydroxypropionaldehyde phosphate and monohydroxyacetone phosphate) could act as 'virtual substrates' of the enzyme. It had been suggested (Rieder & Rose, 1959) that the primary step in the isomerase reaction involves abstraction of a proton from the 2-position of glyceraldehyde phosphate or the 3-position of dihydroxyacetone phosphate, resulting in an enediolate anion. It was expected that the deoxy substrates would be able to suffer this proton abstraction to give an enolate anion, but, lacking the hydroxyl group, could not isomerize (Scheme 1).

It is known that in the enzyme-catalysed reaction of substrate, the proton of the new carbon–hydrogen

bond in the product is very largely solvent-derived (Rieder & Rose, 1959), which means that B–H (in eqn. 1) exchanges rapidly with the solvent. It was therefore expected that for the deoxy compounds, a demonstration of 'virtual substrate' behaviour would be possible by observing enzyme-catalysed exchange of ^2H or ^3H with solvent. Proof of such exchange would not only validate the mechanistic proposals put forward by Rieder & Rose (1959), but would also provide a useful mechanistic 'handle' on half the reaction.

The stability of the two possible 'virtual substrates' was estimated by measuring the release of free phosphate (Briggs, 1922) as a function of time. For monohydroxyacetone phosphate, a linear increase in free phosphate was observed over 12 days, about 4% being hydrolysed after 4 days at pH1.8 and 22°C, and about 14% after 4 days at pH7.8 and 22°C. The interaction of this compound with the enzyme may therefore be studied at neutral pH values during 1 or 2 days with reasonable precision. In contrast with the behaviour of monohydroxyacetone phosphate, 3-propionaldehyde phosphate is subject to facile hydrolysis. At pH1.8 and 22°C, 12% was hydrolysed after 3 days, but at pH7.2 and 22°C, the half-life of this material was about 30min. It was shown that the reaction is first-order in both 3-propionaldehyde phosphate and in HO^- ion, and that the initial product of hydrolysis is acrolein. Analysis either of free phosphate (Briggs, 1922) or of acrolein (by its u.v. absorption in well-stoppered cells) gave similar rates, and the reaction is presumed to involve a base-catalysed β-elimination process. Entirely analogous behaviour is found for β-cyanoethyl phosphate (Lapidot *et al.*, 1963), which has been used as a base-sensitive protecting group for phosphate (Tener, 1961). The lability of 3-hydroxypropionaldehyde phosphate effectively ruled out its use as a 'virtual substrate' for triose phosphate isomerase.

The exchange of the methyl protons of monohydroxyacetone phosphate catalysed by isomerase was investigated both in ^2H_2O (by following the reaction by n.m.r.) and in ^3H_2O (by measuring the incorporation of radioactivity into the substrate). The n.m.r. spectrum of monohydroxyacetone phosphate (0.2M) in ^2H_2O at pH1.8 (meter reading) consisted of a doublet at 5.3τ with J 6Hz, and a singlet at 7.75τ. These are assigned to the two methylene protons and the three methyl protons respectively, and the integrated intensities were accurately in the ratio 3:2. On raising the pH to 7 with NaO^2H in ^2H_2O, no change in the spectrum was observed except for the slight up-field shift of the methylene doublet to 5.5τ.

Incubation at 22°C of this solution showed no significant change in the relative peak intensities over a period of days, although some increase in background signals occurred, presumably from the slow hydrolysis of the ester into monohydroxyacetone, the n.m.r. spectrum of which will be complicated by dimer formation, and Lobry de Bruyn–Alberda van Ekenstein transformation (Speck, 1958) to lactaldehyde. When a small amount of triose phosphate isomerase (to a concentration of approx. 5μN) was added, the ratio of methyl to methylene signals decreased over a period of days (Table 3). After 7 days, the peak ratio had fallen from 3:2 to 1.5:2, showing that about half the methyl protons had exchanged with solvent ^2H_2O. After this time, some 20% of the substrate had been hydrolysed, and about 10% of the enzyme activity had been lost.

These experiments unequivocally demonstrate that triose phosphate isomerase catalyses the exchange of the methyl protons of monohydroxyacetone phosphate with solvent ^2H_2O, though the relative insensitivity of the n.m.r. method precludes any precise estimation of the exchange rate. It is clear, however, that the exchange is very much slower than that of the natural substrate, dihydroxyacetone phosphate.

The rates of exchange of the methyl hydrogen atoms of monohydroxyacetone phosphate catalysed by isomerase and by aldolase were compared by investigating the detritiation reaction. [^3H]Monohydroxyacetone phosphate (see the Experimental section) (approx. 1mM, 3200c.p.m./μmol, in 0.1M-

Table 3. *$^2H_2$ exchange of methyl protons of monohydroxyacetone phosphate*

Monohydroxyacetone phosphate (0.2M) was incubated with triose phosphate isomerase (5μN) at 22°C in ^2H_2O at pH7. For further experimental details see the text.

Time (days)	Methylene doublet intensity (arbitrary units)	Methyl singlet intensity (arbitrary units)	Ratio (singlet/doublet)
0	2.9	4.40	1.51
0.75	3.1	3.7	1.19
4	2.3	2.2	0.96
7	1.8	1.4	0.74
12	1.1	0.75	0.68

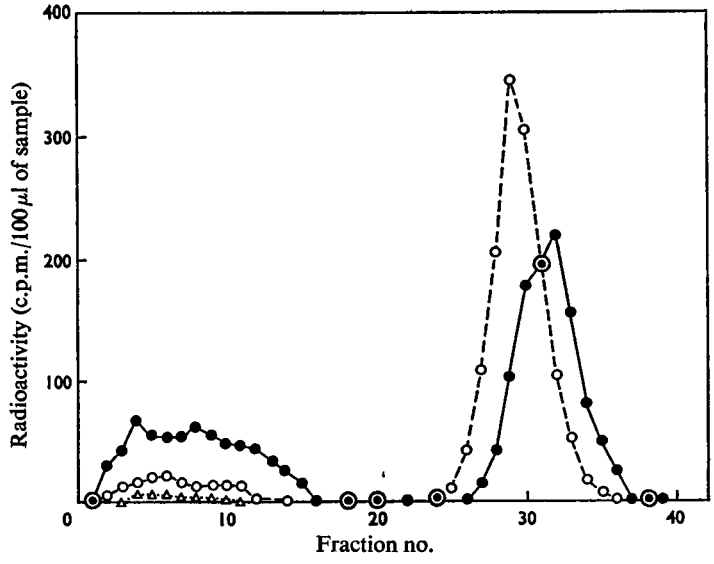


Fig. 3. *Enzyme-catalysed detritiation of [^{3}H]monohydroxyacetone phosphate*

[^{3}H]Monohydroxyacetone phosphate was incubated with aldolase or with isomerase (for experimental details see the text) and the mixture was subjected to chromatography on a column (4cm×1cm) of Dowex 1 (Cl^- form). The ^3H_2O was eluted with 1mM-HCl and the [^{3}H]monohydroxyacetone phosphate with 30mM-HCl. The stepwise change was made around fraction 20. The fraction size was 0.6ml of which 100μl was taken for scintillation counting. The percentage of total radioactivity in the ^3H_2O peak was: with aldolase (—●—), 39.5%; with isomerase (—○—), 9%; and in the absence of enzymes (···△···), 3%.

triethanolamine–HCl buffer, pH7.3, at 37°C) was incubated with isomerase (0.13μN) or with muscle aldolase (0.13μN, assuming a subunit molecular weight of 39500). After 12h the reaction was stopped by rapidly lowering the pH to about 3 and cooling to 0°C. The ^3H_2O that had exchanged out into the medium was then separated from the remaining [^{3}H]monohydroxyacetone phosphate on a column of Dowex 1 (see Fig. 3). It is evident that aldolase is a significantly better catalyst for the exchange reaction than is isomerase. The same was also true for yeast aldolase. To check that the isomerase-catalysed exchange reaction is not due to a small amount of contaminating aldolase, an experiment was performed with a sample of isomerase that had been treated with bromohydroxyacetone phosphate (de la Mare *et al.*, 1972). At the concentrations used, this inhibitor inactivates isomerase completely but is without effect on aldolase. This preparation of catalytically inactive isomerase was also inactive in the exchange reaction. Prolonged incubation with aldolase resulted in the loss of maximally approx. 60% of the substrate radioactivity, indicating that the base-catalysed exchange reaction results in the random labelling of all five carbon-bound hydrogen atoms, and that aldolase (and presumably isomerase) only exchanges the three hydrogen atoms on the methyl group (Rose & O'Connell, 1969).

Triose phosphate isomerase is, therefore, a relatively inefficient catalyst for the exchange of the methyl hydrogen atoms of monohydroxyacetone phosphate with solvent. Rose & O'Connell (1969) have reported that monohydroxyacetone phosphate is a 'virtual substrate' for aldolase, and showed that the rate of exchange was some 17% of that with the natural substrate, dihydroxyacetone phosphate. The relative inefficiency in the exchange reaction of isomerase (the exchange reaction is about 10^4-fold slower than the corresponding reaction with dihydroxyacetone phosphate) is curious. But these two enzymes, despite the formal similarity in the first steps of their postulated mechanisms (Rose, 1962) do differ in their behaviour towards other substrate analogues, for instance bromo- and chloro-hydroxyacetone phosphate (see de la Mare *et al.*, 1972), and it is clear that the details of these enzyme–substrate interactions are not straightforward.

Kinetics of the triose phosphate isomerase reaction

The validity of the two coupled enzyme assays used to follow the forward and reverse reactions

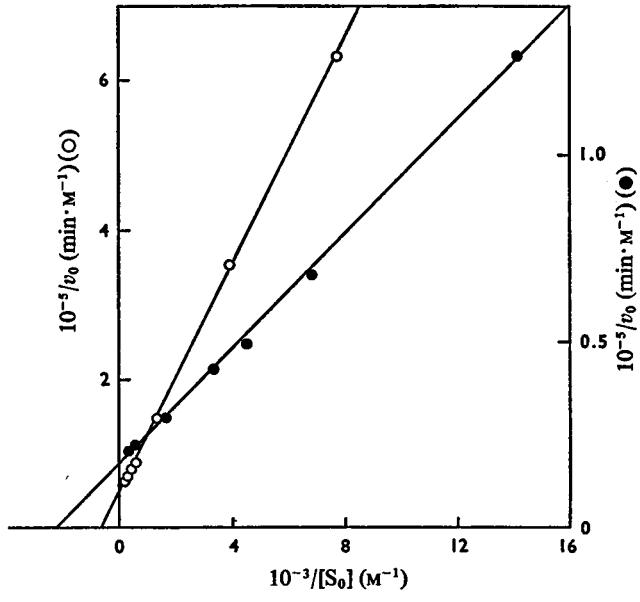


Fig. 4. *Lineweaver–Burk plots for the triose phosphate isomerase-catalysed reaction of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate*

Reactions were carried out in 0.1M-triethanolamine–HCl buffer at 30°C, at pH7.27 for dihydroxyacetone phosphate (○), and at pH7.42 for glyceraldehyde 3-phosphate (●). v_0 is the initial velocity at initial substrate concentration $[S_0]$.

catalysed by triose phosphate isomerase are discussed fully by Plaut & Knowles (1972). The experiments reported here were performed over much wider substrate concentration ranges than those used by Plaut & Knowles (1972), to check the existence of inhibition by excess of substrate, a phenomenon observed with the rabbit muscle enzyme by Snyder & Lee (1966). The checks on the validity of the assay described by Plaut & Knowles (1972) were extended to cover the higher substrate concentrations used, to ensure both that the higher product fluxes could be eliminated quickly enough by the coupling dehydrogenase, and that the high substrate concentrations were without effect on the coupling enzyme. When dihydroxyacetone phosphate is substrate, it is not difficult to maintain the conditions such that the isomerization is rate-limiting. When glyceraldehyde 3-phosphate is substrate, however, it is very easy to observe what superficially resembles inhibition by excess of substrate. When the dependence of the reaction rate on isomerase concentration is checked [see Fig. 3(*b*) of Plaut & Knowles (1972)] at high glyceraldehyde phosphate concentration, and isomerase concentrations are limited to those within the linear region, the double-reciprocal plots for the isomerization of glyceraldehyde phosphate are linear over the whole substrate concentration range (Fig. 4). Snyder & Lee (1966) have reported inhibition by excess of substrate for both muscle and liver triose phosphate isomerases from the horse. We have not checked the chicken enzyme by using the non-continuous assay method employed by Snyder & Lee (1966), but we see no reason to doubt the validity of the results from the coupled enzyme assay method used here.

The kinetic parameters obtained from the results shown in Fig. 4 must be corrected for the effect of arsenate on the isomerase reaction (an effect first noted by Burton & Waley, 1968*b*). The K_i for competitive inhibition by arsenate of the reaction with dihydroxyacetone phosphate as substrate, under the assay conditions used here (I0.1, 30°C, pH7.5) was found to be 11.1mM [Burton & Waley (1968*b*) report 5.5mM at 25°C for the rabbit muscle enzyme]. The apparent K_m for dihydroxyacetone phosphate (1.5mM) was corrected by using this value, to give 0.97mM. The $k_{cat.}$ in this direction was 2.59×10^4min^{-1}. With glyceraldehyde phosphate as substrate, the Michaelis parameters were K_m 0.47mM and $k_{cat.}$ 2.56×10^5min^{-1}. Application of the Haldane relationship $[K_{eq.}=(k_{cat.}/K_m)/(K'_m/k'_{cat.})$, where the primed parameters relate to the reverse reaction]

gives a value for $K_{eq.}$ of 20.4 (at 30°C). This may be compared with a value of 22 (at 25°C) obtained similarly by Burton & Waley (1968*b*), and an average value of 22.0±0.25 from 30 direct measurements of the equilibrium constant at 38°C by Veech *et al.* (1969).

We gratefully acknowledge the work of Mr. A. D. Bisset and Mr. G. Midgley on the thiol titrations, the technical assistance of Mr. J. Law, and financial support from the Science Research Council and the Medical Research Council. This is a contribution from the Oxford Enzyme Group.

References

Baer, E. (1952) *Biochem. Prep.* **2**, 96
Ballard, S. A., Geyer, B. P. & Mortimer, R. H. (1951) *Brit. Patent* 645,956; cited in *Chem. Abstr.* (1951) **45**, 7589h
Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C. & Pogson, C. I. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 151
Briggs, A. P. (1922) *J. Biol. Chem.* **53**, 13
Burton, P. M. & Waley, S. G. (1968*a*) *Exp. Eye Res.* **7**, 189
Burton, P. M. & Waley, S. G. (1968*b*) *Biochim. Biophys. Acta* **151**, 714
Czok, R. & Bücher, T. (1960) *Advan. Protein Chem.* **15**, 315
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404
de la Mare, S., Coulson, A. F. W., Knowles, J. R., Priddle, J. D. & Offord, R. E. (1972) *Biochem. J.* **129**, 321
Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70
Fahey, R. C., Kolb, E. & Harris, J. I. (1971) *Biochem. J.* **124**, 77P
Fischer, R. F. & Smith, C. W. (1959) *U.S. Patent* 2857422; cited in *Chem. Abstr.* (1959) **53**, 5203i
Gilman, A., Philips, F. S., Koelle, E. S., Allen, R. P. & St. John, E. (1946) *Amer. J. Physiol.* **147**, 115
Johnson, L. N. & Waley, S. G. (1967) *J. Mol. Biol.* **29**, 321
Johnson, L. N. & Wolfenden, R. (1970) *J. Mol. Biol.* **47**, 93
Krietsch, W. K. G., Pentchev, P. G., Klingenbürg, H., Hofstätter, T. & Bücher, T. (1970*a*) *Eur. J. Biochem.* **14**, 289
Krietsch, W. K. G., Pentchev, P. G., Machleidt, W. & Klingenbürg, H. (1970*b*) *FEBS Lett.* **11**, 137
Krietsch, W. K. G., Pentchev, P. G. & Klingenbürg, H. (1971) *Eur. J. Biochem.* **23**, 77
Lapidot, A., Samuel, D. & Silver, B. (1963) *Chem. Ind. (London)* 468
Lappin, G. R. & Clark, L. C. (1951) *Anal. Chem.* **23**, 541
Lee, E. W., Barriso, J. A., Pepe, M. & Snyder, R. (1971) *Biochim. Biophys. Acta* **242**, 261
McGilvery, R. W. (1953) *J. Biol. Chem.* **200**, 835
McVittie, J. D., Esnouf, M. P. & Peacocke, A. R. (1972) *Eur. J. Biochem.* in the press
Meeks, J. C., Willson, D. L. & Gaines, R. D. (1968) *Phytochemistry* **7**, 2095
Meyer-Arendt, E., Beisenherz, G. & Bücher, T. (1953) *Naturwissenschaften* **40**, 59
Norton, I. L., Pfuderer, P., Stringer, C. D. & Hartman, F. (1970) *Biochemistry* **9**, 4952
Pihl, A. & Lange, R. (1962) *J. Biol. Chem.* **237**, 1356
Plaut, B. & Knowles, J. R. (1972) *Biochem. J.* **129**, 311
Rieder, S. V. & Rose, I. A. (1959) *J. Biol. Chem.* **234**, 1007
Rose, I. A. (1962) *Brookhaven Symp. Biol.* **15**, 293
Rose, I. A. & O'Connell, E. L. (1969) *J. Biol. Chem.* **244**, 126
Scopes, R. K. (1968) *Biochem. J.* **107**, 139
Sellinger, O. Z. & Miller, O. N. (1958) *Biochim. Biophys. Acta* **29**, 74
Shapiro, A. L., Vinuela, E. & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun.* **28**, 815
Snyder, R. & Lee, E. W. (1966) *Arch. Biochem. Biophys.* **117**, 587
Speck, J. C. (1958) *Advan. Carbohyd. Chem.* **13**, 63
Tener, G. M. (1961) *J. Amer. Chem. Soc.* **83**, 159
Trentham, D. R., McMurray, C. H. & Pogson, C. I. (1969) *Biochem. J.* **114**, 19
Turner, D. H., Blanch, E. S., Gibbs, M. & Turner, J. F. (1965) *Plant Physiol.* **40**, 1146
Veech, R. I., Raijman, L., Dalziel, K. & Krebs, H. A. (1969) *Biochem. J.* **115**, 837
Wolfenden, R. (1969) *Nature (London)* **223**, 704