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Isolation and Characterization of Rabbit Muscle Triose Phosphate Isomerase*

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ABSTRACT: Rabbit muscle triose phosphate isomerase has been purified to homogeneity as judged by analytical ultracentrifugation and disc gel electrophoresis. The enzyme has a molecular weight of 52,900 by sedimentation equilibrium, 56,000 by amino acid composition, and 53,000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Data from carboxypeptidase digestion of the enzyme are consistent with two identical subunits having a C-terminal sequence of -(Val,Asp,Phe,Ile)-Asn-Ile-Ala-Lys-Gln. Electrofocusing of triose phosphate isomerase reveals the presence of two major and two minor species having similar specific activities.

Recent studies (Hartman, 1970a,b) on the active site of rabbit muscle triose phosphate isomerase (D-glyceraldehyde 3-phosphate:ketol isomerase, EC 5.3.1.1) required large amounts of the enzyme. The difficulty in obtaining sufficient quantities by a previously published method (Czok and Bücher, 1960) and the heterogeneity of commercial material prompted the development of the isolation procedure described in our present report. The purified enzyme has been characterized with respect to amino acid composition, molecular weight, and subunit structure.

Materials and Methods

Frozen, deboned muscle tissue from the back and hind legs of young (8–12 weeks) rabbits was purchased from Pel-Freez Biologicals, Inc. NADH, DL-glyceraldehyde 3-phosphate, α-glycerophosphate dehydrogenase, and rabbit muscle triose phosphate isomerase were obtained from the Sigma Chemical Co. Rabbit muscle triose phosphate isomerase was also obtained from Boehringer Mannheim Corp. Carboxypeptidase A was a product of Worthington Biochemical Corp. Ammonium sulfate (Ultra Pure biological grade) was purchased from Schwarz BioResearch, Inc.

Protein Concentration. The method of Lowry *et al.* (1951) with human serum albumin as the standard or spectrophotometric measurements at 280 nm were used to determine protein concentrations. The $\epsilon_{1\,cm}^{1\%}$ at 280 nm of purified triose phosphate isomerase was determined as described below.

Enzyme Assays. Triose phosphate isomerase was assayed at 24° by the method of Beisenherz (1955). The assay mixture (3 ml) contained 0.15 mM NADH, 1 mM DL-glyceraldehyde 3-phosphate, 28 μg of α-glycerophosphate dehydrogenase, 0.3 mM EDTA, and 20 mM triethanolamine hydrochloride (pH 7.9). The reaction was initiated by the addition of a quantity of triose phosphate isomerase such that the change in A per minute at 340 nm did not exceed 0.2. One unit of activity is defined as the conversion of 1 μmole of glyceraldehyde 3-phosphate into dihydroxyacetone phosphate per min and represents a decrease in A_{340nm} of 2.07 optical density units per min.

Extinction Coefficient. Two samples of triose phosphate isomerase at about 8 mg/ml were dialyzed exhaustively (three changes during 48 hr) against 0.01 M ammonium acetate (pH 6.4). Aliquots of the dialyzed samples were diluted 20-fold into various buffers (0.05 M Tris-hydrochloride, pH 7.5; 0.1 M sodium phosphate, pH 6.0, 7.0, and 8.0; 0.1 M sodium cacodylate, pH 6.0). The A at 280 nm of these solutions were identical. Samples (3 ml) of the undiluted, dialyzed protein solutions were lyophilized to dryness in preweighed vials, redissolved in 5 ml of water, and again lyophilized. The vials were then heated in a vacuum desiccator to constant weight, from which the $\epsilon_{1\,cm}^{1\%}$ of the two samples was calculated as 13.07 and 13.18, respectively. Values of 11.0 and 12.1 have been reported for commercial rabbit muscle triose phosphate isomerase (Hartman, 1968; Coulson *et al.*, 1970).

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Amino Acid Composition. Protein was hydrolyzed at 110° for 24, 48, and 72 hr in sealed, evacuated (<50 μ) tubes with 6 N HCl containing 0.01 M β-mercaptoethanol. Before evacuation, the solutions were flushed with nitrogen to remove dissolved oxygen. The hydrolysates were concentrated to dryness on a rotary evaporator and analyzed on a Beckman Model 120C amino acid analyzer according to Spackman *et al.* (1958).

Tryptophan in the unhydrolyzed protein was determined spectrophotometrically by the method of Edelhoch (1967). Free sulfhydryl groups in triose phosphate isomerase were measured by a modification of Ellman's (1959) procedure. Into cuvets containing 2.3-ml total of 6 M guanidine hydrochloride, 0.1 M sodium phosphate, and 5 mM EDTA (pH 8.0) were added 0.5–1.0 mg (0.1 ml) of triose phosphate isomerase and 0.1 ml of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid). The increase in A at 412 nm was complete within 30 sec, and the sulfhydryl concentration was calculated using ϵ 13,600.

Electrophoresis. Polyacrylamide disc gel electrophoresis, polyacrylamide gel electrophoresis in sodium dodecyl sulfate, and electrofocusing on polyacrylamide gels were carried out with the analytical apparatus from Canal Industrial Corp. Polyacrylamide disc gel electrophoresis was conducted on standard 7% gels according to the manufacturer's instructions. Gels were stained with Amido Schwarz and destained electrophoretically.

Polyacrylamide electrophoresis (10% gels) in sodium dodecyl sulfate to determine the subunit molecular weight of triose phosphate isomerase was accomplished by the method of Weber and Osborn (1969). The ammonium persulfate concentration in the gel solution was decreased from 0.75 to 0.25 mg per ml.

Electrofocusing on polyacrylamide gels (7.5%) was conducted by the method of Hayes and Wellner (1969) with slight modification. The final riboflavin content in the gel solution was decreased from 0.0015 to 0.001%, since at the higher concentration gelling did not occur. Focusing time was decreased from 9 hr to 3–4 hr. Gels were fixed, washed, and stained with coomassie brilliant blue as reported (Hayes and Wellner, 1969), but background stain was removed by washing with acetic acid–ethanol–water (2:15:13, v/v) (Riley and Coleman, 1968).

Electrofocusing was also conducted in a sucrose gradient (Vesterberg and Svensson, 1966). An LKB apparatus was used according to the manufacturer's instructions.

C-Terminal Analysis. Triose phosphate isomerase was oxidized with performic acid by the method of Hirs (1956). After lyophilization, the oxidized protein was dissolved in 6 M guanidine hydrochloride at a concentration of about 10 mg/ml and dialyzed against 0.2 M *N*-ethylmorpholinium acetate (pH 8.5). During dialysis protein did not precipitate. The protein concentration was diluted to 3 mg/ml with the buffer used in dialysis, and to 10 ml of this solution at 40° was added 0.3 mg (0.1 ml) of carboxypeptidase A. The digestion mixture was maintained at 40°, and periodically 1-ml aliquots were deproteinized with 0.1 ml of 10% trichloroacetic acid. After centrifugation (10,000g for 5 min), 0.4 ml of the supernatant was analyzed on the amino acid analyzer, using a lithium buffer system (prepared according to the Beckman 120C instruction manual) in order to resolve serine, glutamine, and asparagine. An identical solution containing carboxypeptidase A, but without triose phosphate isomerase, served as control. Corrections were made for free amino acids found in the control digest.

Analytical Ultracentrifugation. Sedimentation equilibrium and sedimentation velocity experiments were run on a Spinco Model E equipped with RTIC, schlieren, and interference optical systems, and adjustable optical components. The Rayleigh optical system was aligned according to Gropper (1964). All sedimentation velocity experiments were done in 12-mm 2° Kel F centerpieces at 59,780 rpm and 20°. The equilibrium measurements were made in double-sector-filled-epon centerpieces for 26 hr at 20,410 rpm at 20°, using 3- or 4-mm column heights. The meniscus depletion technique of Yphantis (1964) was used, with overspeeding to hasten equilibrium time. All solutions were exhaustively dialyzed against solvent before use, and the cells were aligned in the rotor, using the microscope cell aligner. A blank run using the same untorqued cells was used as a base line, as suggested by Yphantis (1964).

Results

Purification of Triose Phosphate Isomerase. Unless stated otherwise, all operations were performed at 4°, and aqueous solutions were prepared with glass-distilled water.

Extraction. Partially thawed tissue (450 g) was ground twice in a chilled meat grinder. The ground tissue was divided into three equal portions, each of which was homogenized with 200 ml of 1.5 mM EDTA (pH 5.2) for 1 min at high speed in a Waring blender. The resulting homogenates were pooled, stirred for 30 min, and centrifuged for 15 min at 10,400g. The supernatant was strained through glass wool. The sediment was rehomogenized (400 ml of 1.5 mM EDTA), stirred, and centrifuged as above. The combined muscle extracts were adjusted to 930 ml with 1.5 mM EDTA, and β-mercapothethanol was added to 1.0 mM.

Acetone Fractionation. During 3 min and with continuous stirring, 500 ml of acetone (−15°) was added to the muscle extract (final acetone concentration is 35%). The temperature of the mixture did not rise above 7°. After centrifugation for 10 min at 10,400g, the precipitate was discarded and acetone (183 ml/l of 35% acetone supernatant) was added, with stirring, to the supernatant to a final concentration of 45%. Best results were obtained when this addition took about 30 min and the temperature of the mixture did not rise above 4°. The 45% acetone mixture was centrifuged as before. The precipitate was discarded, and acetone (195 ml/l. of 45% acetone supernatant) was added to the supernatant, as described in the preceding step, to a final concentration of 54%. This fraction was centrifuged, and the supernatant was discarded.

Heat Treatment. The 54% acetone precipitate was dissolved in 0.05 M sodium phosphate–3 mM EDTA–1 mM β-mercaptoethanol (pH 7.0) to a final volume of 100 ml. The slightly turbid solution was stirred gently in a 100 × 50 mm crystallizing dish with a magnetic stirrer at 25° for 12 hr to remove traces of acetone. The volume was readjusted to 100 ml with the same buffer, and the solution was brought to 51° during 10 min by gentle swirling in a constant-temperature bath and maintained at the elevated temperature for 30 min. Precipitated material was removed by centrifugation at 15,900g for 15 min.

Sephadex G-100. The supernatant (90 ml) from the pre-

TABLE I: Purification of Rabbit Muscle Triose Phosphate Isomerase.

Fraction	Vol (ml)	Total Protein (mg)	Total Act. (units)	Sp Act. (units/mg)	Yield (%)
Extract	930	18,600[a]	3,200,000	170	100
54% acetone precipitate	100	4,400[a]	2,400,000	545	75
Heat treated	96	2,250[b]	2,100,000	950	67
Sephadex G-100 pool	206	608[b]	1,810,000	2980	57
Crystals from ammonium sulfate (3.6–3.7 *m*)		169[b]	1,158,000	6900	37
After SE-Sephadex and repeated crystallization		85[b]	663,000	7800	21

[a] Based on Lowry determinations (see Materials and Methods). [b] Based on *A* at 280 nm (see Materials and Methods).

ceding step was passed through a 6.0 × 185 cm column of Sephadex G-100 equilibrated with a solution containing 0.05 M Tris-hydrochloride, 3 mM EDTA, and 1 mM β-mercapothethanol (pH 7.5). Triose phosphate isomerase eluted at 1550–1800 ml (Figure 1). Fractions containing enzyme with a specific activity greater than 1300 were pooled and dialyzed against Tris buffer (as used for gel filtration) saturated with ammonium sulfate. The precipitated protein was collected by centrifugation at 15,900*g* for 20 min.

Crystallization. The precipitate was redissolved in the same Tris buffer (no ammonium sulfate) to a final protein concentration of 4.25 mg/ml (final volume, 138 ml), and solid ammonium sulfate (62 g) was added to 3.4 *m*. Precipitated protein was removed by centrifugation at 15,900*g* for 20 min. The supernatant was brought to 3.5 *m* ammonium sulfate (1.8 g added) and the precipitated protein again removed by centrifugation at 15,900*g* for 20 min. The supernatant was brought to 3.6 *m* ammonium sulfate (1.8 g added) and allowed to stand undisturbed. After 3 days, the needle-shaped crystals of triose phosphate isomerase were collected by centrifugation. A second crop of crystals was obtained by increasing the ammonium sulfate concentration of the supernatant to 3.7 *m* (1.8 g added).

SE-Sephadex Chromatography. Crystalline triose phosphate isomerase from the preceding step was dialyzed against 0.05 M sodium phosphate containing 3 mM EDTA (pH 5.5) and placed on a 2 × 57 cm column of SE-Sephadex C-50 equilibrated with the same phosphate buffer. The column was eluted with a linear gradient of sodium chloride (0–0.5 M) in the phosphate buffer (Figure 2). The eluted enzyme was precipitated as in the Sephadex G-100 step and crystallized as in the preceding step.

A summary of the isolation procedure is given in Table I.

Characterization of Triose Phosphate Isomerase (after SE-Sephadex Chromatography). HOMOGENEITY. The enzyme (isolated as described) sediments as a single boundary in the analytical ultracentrifuge (Figure 3). The concentration dependence of the sedimentation constant, shown in Table II, leads to a value of $s^0_{20,w} = 3.95$ S at infinite dilution.

As judged by disc gel electrophoresis, the enzyme is free of minor constituents present in commercial preparations (Figure 4). Crystalline triose phosphate isomerase obtained after Sephadex G-100 chromatography and crystallization, but before SE-Sephadex chromatography, is contaminated with a single, minor species (Figure 4).

The enzyme which travels as a single band during disc gel electrophoresis is resolved by electrofocusing into multiple components. Using a pH 5–8 gradient in polyacrylamide, we found one major and two minor bands (Figure 5). Electro-

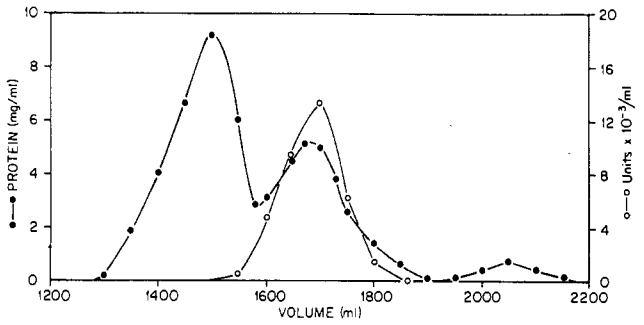

FIGURE 1: Purification of triose phosphate isomerase on Sephadex G-100. Experimental details are described in text.

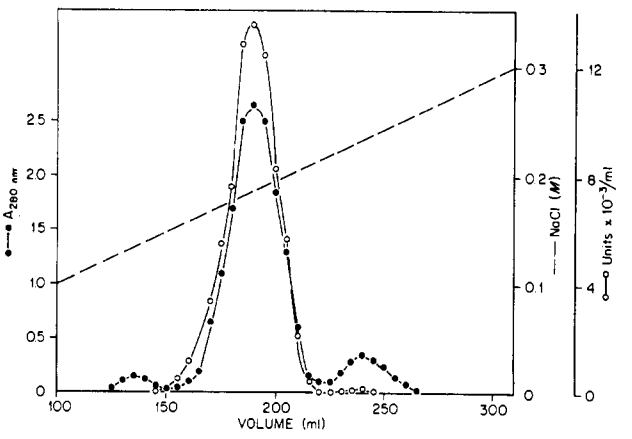

FIGURE 2: Purification of triose phosphate isomerase by ion-exchange chromatography on SE-Sephadex. Experimental details are described in text.

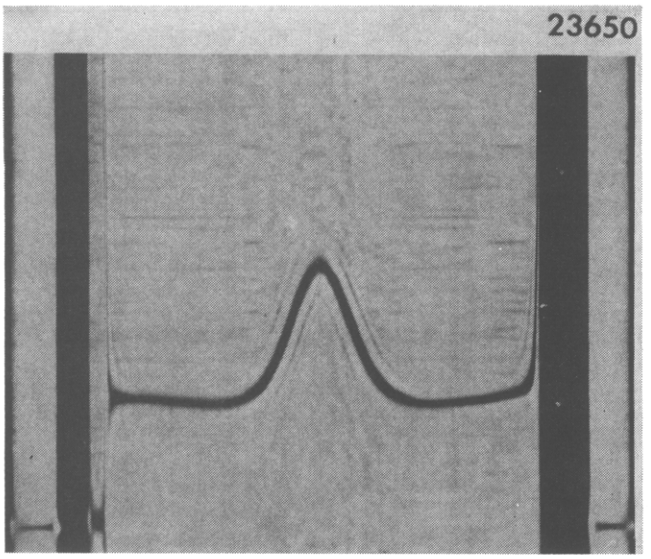

FIGURE 3: Schlieren pattern from triose phosphate isomerase at 10 mg/ml photographed 125 min after reaching 59,780 rpm. Temperature, 20°; bar angle, 60°; solvent, 0.02 M Tris-HCl (pH 7.5)–0.2 M NaCl–1 mM EDTA.

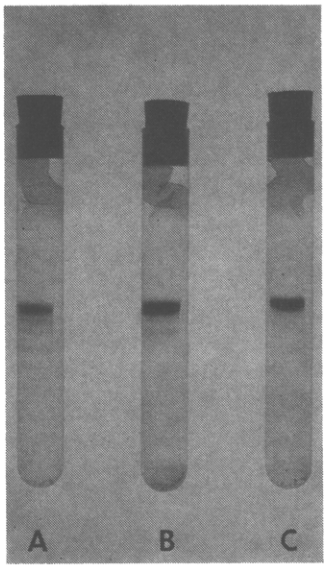

FIGURE 4: Disc gel electrophoresis at pH 9.5 of triose phosphate isomerase. Direction of migration is toward bottom of tube (anode). (A) Commercial enzyme from Sigma Chemical Co.; (B) our preparation before SE-Sephadex chromatography; (C) our preparation after SE-Sephadex chromatography.

focusing in a sucrose stabilized, pH 5–8 gradient, formed from the same lot of ampholyte as employed in the polyacrylamide experiment, partially resolves the major band into two components (Figure 5). Although the resolution is slight, identical results were obtained in three separate experiments. The separation was not improved by altering the duration of the applied potential. Although slight inactivation may have occurred during electrofocusing, the specific activities of the isoenzymes (present at a ratio of 1:3:12:12) are similar—6800 units/mg for the minor components and 7300 units/mg for the major components, as compared to 7500 units/mg for the preparation before electrofocusing. The apparent isoelectric points of the minor species are pH 5.55 and 5.95 and of the major species are pH 6.75 and 6.85.

Amino Acid Composition. The amino acid compositions of our preparations and of commercial preparations as reported by Burton and Waley (1966) are given in Table III. Based on methionine, which is present in the least amount, the minimal molecular weight of triose phosphate isomerase is 14,000. Assuming 2 identical subunits with 2 methionyl residues each, the actual molecular weight of the enzyme is 56,000.

TABLE II: Concentration Dependence of Sedimentation Rate of Triose Phosphate Isomerase.

Protein Concn (mg/ml)	$s_{20,w}$ (S)
1	3.89
2	3.83
4	3.77
5	3.79
6	3.65
8	3.58
10	3.59

Molecular Weight. The weight-average molecular weight of triose phosphate isomerase, determined in three separate sedimentation equilibrium experiments in which protein concentrations were 2.0, 0.5, and 0.2 mg per ml, is 52,800, 52,600, and 53,300, respectively. Since there is little concen-

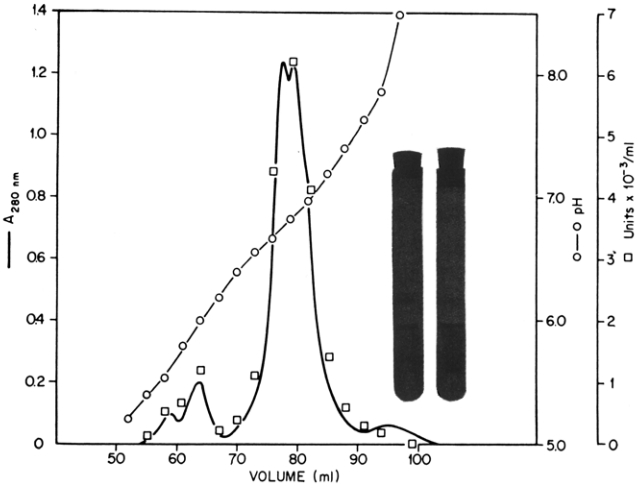

FIGURE 5: Electrofocusing of triose phosphate isomerase (15 mg, specific activity of 7500 units/mg) in a sucrose gradient. The procedure was conducted on an Ampholine column (110 ml) according to the instructions provided by LKB instruments, using a narrow range (pH 5–8) carrier ampholyte at a concentration of 1%. Focusing was continued at 600 V for 72 hr. The A_{280nm} profile represents a direct tracing obtained from monitoring the column effluent. The activity profile represents assays on individually collected fractions (3 ml). The insert is a photograph of analytical electrofocusing experiments carried out on polyacrylamide gels. The sample on the left of the photograph is a commercial enzyme (Sigma) and on the right is a portion of the same preparation that was focused in the liquid system. The pH increases from top to bottom of the gels.

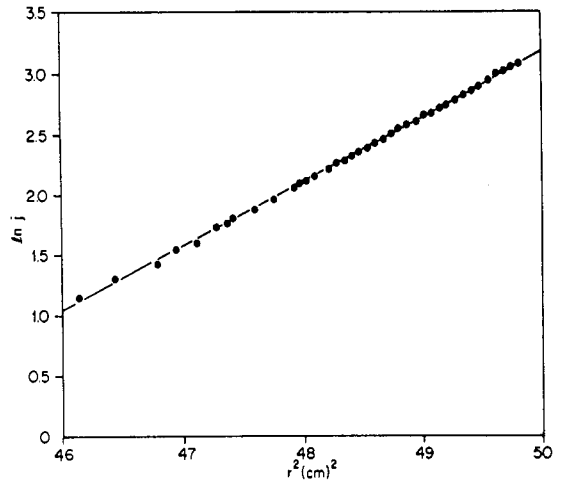

FIGURE 6: Data from equilibrium sedimentation of triose phosphate isomerase 26 hr after reaching 20,410 rpm. Solvent and temperature are the same as in Figure 3.

tration dependence of molecular weight over the range used, the best value for the molecular weight is probably the average from the three determinations—52,900 ± 2000. Data from one experiment are shown in Figure 6. A partial specific volume ($\bar{v}$) of 0.737 ml/g, determined from the amino acid composition, was used in the calculations. With a $\bar{v}$ of 0.737 ml/g and an $s^0_{20,w}$ of 3.95 S, triose phosphate isomerase has a diffusion coefficient (D) of 6.9×10^{-7} cm² sec⁻¹ and a frictional ratio (f/f_0) of 1.25.

The subunit molecular weight is 26,500, based on polyacrylamide electrophoresis in sodium dodecyl sulfate (Figure 7). Thus, native enzyme is composed of 2 subunits.

C-Terminal Sequence. The C-terminal sequence of triose phosphate isomerase deduced from the rate of release of amino acid upon treating the enzyme with carboxypeptidase A (Figure 8) is -(Val,Asp,Phe,Ile)-Asn-Ile-Ala-Lys-Gln. The subunit molecular weight of the enzyme, based on the amounts of glutamine, lysine, alanine, and asparagine released, is 26,000–29,000, which agrees with that found by electrophoresis and also indicates that triose phosphate isomerase contains 2 subunits.

TABLE III: Amino Acid Composition of Triose Phosphate Isomerase.

Amino Acid	No. of Residues/ Methionine		No. of Residues/ 53,000 g	
	This Report[a]	Burton and Waley (1966)	This Report	Burton and Waley (1966)
Lysine	11.3 (0.32)	11.1	42.8	48.1
Histidine	1.9 (0.10)	2.5	7.6	11.1
Arginine	4.25 (0.09)	4.2	16.1	19.7
Aspartic acid	11.4 (0.08)	9.9	43.2	43.1
Threonine[b]	8.0 (0.10)	7.1	30.3	30.8
Serine[b]	6.5 (0.15)	5.6	24.6	27.1
Glutamic acid	15.2 (0.11)	11.9	57.5	51.8
Proline	4.6 (0.18)	3.4	17.4	14.8
Glycine	13.1 (0.10)	12.4	49.6	53.0
Alanine	15.3 (0.14)	11.4	57.9	49.3
Valine	12.4 (0.25)	9.7	46.9	41.9
Methionine	1.0	1.0	3.8	4.9
Isoeleucine	6.7 (0.17)	5.5	25.4	23.4
Leucine	8.1 (0.10)	7.6	30.7	33.3
Tyrosine	2.1 (0.018)	2.2	7.9	9.9
Phenylalanine	4.0 (0.10)	3.8	15.1	16.0
Cysteine	2.8[d] (0.11)	2.2	10.6[d] (9.2)[e]	9.9
Tryptophan[c]		1.0	9.5	4.9

[a] Number of residues reported represents averages from five separate determinations, and the values in parentheses are standard deviations. [b] Serine and threonine were corrected for destruction during hydrolysis by extrapolation to zero time. [c] Tryptophan was determined in unhydrolyzed protein as described in Materials and Methods section. [d] Cysteine was determined as cysteic acid after oxidation of the protein with performic acid (Hirs, 1956) or by including dimethyl sulfoxide in the hydrolysis mixture (Spencer and Wold, 1969). [e] Number of free sulfhydryl groups determined as described in Materials and Methods section.

Discussion

Although commercially available rabbit muscle triose phosphate isomerase has been characterized previously, several considerations suggested the need for reevaluating the chemical and physical properties of the enzyme. Commercial preparations have varying specific activities and are heterogeneous as judged by disc gel electrophoresis. The amino acid composition of commercial triose phosphate isomerase determined in this laboratory did not entirely agree with that reported by Burton and Waley (1966). The molecular weight of triose phosphate isomerase has been reported as 53,000 from crystallographic data (Johnson and Waley, 1967), 48,000 (unpublished data of P. Esnouf and J. Jesty cited by Coulson *et al.*, 1970), and 60,000 (Johnson and Waley, 1967) from equilibrium ultracentifugation, 53,000 (assuming 2 subunits) from polyacrylamide electrophoresis in sodium dodecyl sulfate (unpublished data of Katze and Knowles cited by Coulson *et al.*, 1970), and 43,000 from gel filtration (Burton and Waley, 1966), the latter number having been retracted (Burton and Waley, 1968). Unfortunately, very little data, from which these values were calculated, have been recorded and are thus difficult to evaluate. The advantages of working with an enzyme of known and reproducible composition and whose isolation can be repeated in other laboratories necessitated devising the purification summarized in Table I. One isolation procedure for rabbit muscle triose phosphate isomerase has been described previously (Czok and Bücher, 1960), but it appeared impractical because of the lack of experimental detail and the use of a preparative electrophoretic step.

We have isolated triose phosphate isomerase ten times during the past year, and the specific activities (7500 units/mg ±5%) of these preparations are about 10% greater than those of the best commercial preparations, which have varied from 5500 to 6900 units per mg. The enzyme is homogeneous by

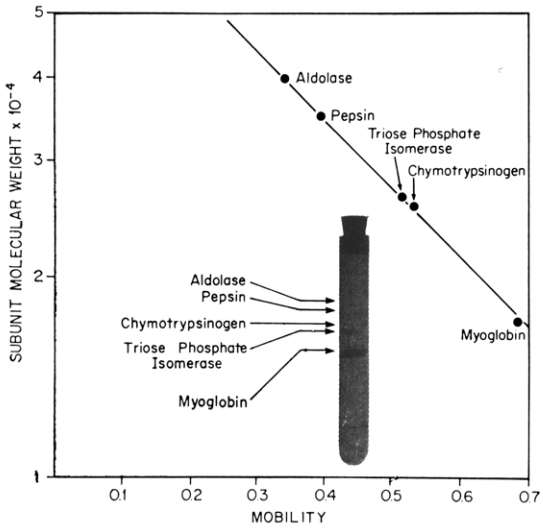

FIGURE 7: Subunit molecular weight of triose phosphate isomerase as determined by polyacrylamide electrophoresis in sodium dodecyl sulfate. The inserted photograph is from the run that provided the plotted data. Experimental details are given in the Materials and Methods section.

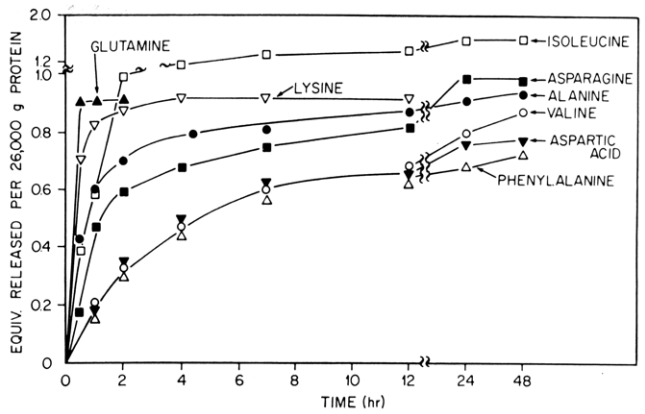

FIGURE 8: Time course of amino acids released from performic acid oxidized triose phosphate isomerase upon incubation with carboxypeptidase A. Experimental conditions are given in the Materials and Methods section.

disc gel electrophoresis (Figure 4) and by sedimentation velocity (Figure 3). Burton and Waley (1966) have reported that some commercial preparations contain a single protein band by disc gel electrophoresis, but we have never obtained such pure material from commercial sources.

There are a number of discrepancies in the amino acid composition of our preparations as compared to commercial preparations reported previously (Burton and Waley, 1966). Some of these differences may in part be due to actual variations in chemical composition of the two samples. Differences in the tryptophan content may reflect the assay used. Burton and Waley (1966) used the chemical method of Spies and Chambers (1949), whereas we used the direct spectrophotometric method of Edelhoch (1967). Actually, the peptide mapping data of Burton and Waley (1966) suggested 4 or 5 tryptophanyl residues per subunit, which is consistent with the 10 residues per molecule reported here. The number of free sulfhydryl groups found in the unhydrolyzed protein approximates the number measured as cysteic acid, but the data do not entirely exclude the possibility of a single disulfide bond in native triose phosphate isomerase.

Calculated from amino acid analyses and equilibrium ultracentrifugation, the molecular weight of triose phosphate isomerase isolated in the present study is 56,000 and 53,000, respectively. The subunit molecular weight (26,500) obtained by polyacrylamide electrophoresis in sodium dodecyl sulfate is in excellent agreement with unpublished data from the same technique cited by Coulson *et al.* (1970) and demonstrates the presence of 2 subunits in the native enzyme. The value obtained from amino acid analyses may be somewhat high since methionine, the amino acid present in the smallest amount and upon which the calculation is therefore based, is susceptible to oxidation to its sulfoxide during hydrolysis. To minimize this undesirable side reaction, β-mercaptoethanol was included in the hydrolysis mixture, and the protein samples were flushed with nitrogen before evacuation. Variations in molecular weights, obtained by sedimentation equilibrium, of 53,000 reported here and 60,000 (Johnson and Waley, 1967) and 48,000 (unpublished data of P. Esnouf and J. Jesty cited by Coulson *et al.*, 1970) reported elsewhere may in part be due to the use of different values for the partial specific volume. We have used 0.737 ml/g calculated from the amino acid composition. Other values for the partial specific volume have not been published.

Based on quantitative N-terminal and qualitative C-terminal determinations, disc electrophoresis in 8 M urea, and number of tryptic peptides containing certain amino acids, Burton and Waley (1966, 1968) concluded that commercial triose phosphate isomerase contains 2 very similar, if not identical, subunits. As an extension of their studies, we quantitatively determined the amino acids released by carboxypeptidase A and confirm the presence of glutamine in the C-terminal position. Whereas with native enzyme in buffered urea, only glutamine was released (Burton and Waley, 1968), in the present study with performic acid oxidized triose phosphate isomerase, eight other residues were removed from the C-terminal end. The time course of their appearance is consistent with the C-terminal sequence of -(Val,Asp,Phe, Ile)-Asn-Ile-Ala-Lys-Gln (Figure 8). The subunit molecular weight calculated from the residues which reached a constant contration in the digestion mixture (glutamine, lysine, alanine, and asparagine) varies from 26,000 to 29,000. The residues which did not reach maximal concentrations during the digestion period were, however, released to an extent greater than 0.5 mole/26,000 g of protein. There is thus no indication of one chain being degraded further than the other, and the data are entirely consistent with the two chains being identical in the C-terminal region.

In commercial triose phosphate isomerase, Burton and Waley (1966) have detected by starch gel electrophoresis two minor and one major component with enzymic activity, and Lee and Snyder (1970), using polyacrylamide electrophoresis, have reported the presence of five isoenzymic species at a ratio of 7:15:6:2:1. We have used electrofocusing to determine whether highly purified triose phosphate isomerase exists in isoenzymic forms. If the procedure is carried out in polyacrylamide gels, one major and two minor components are seen (Figure 5). Electrofocusing in sucrose gradients also reveals two minor components, but the major component is

partially resolved into two components (Figure 5). Such poorly resolved species in polyacrylamide would likely appear as a single band when visualized with a protein stain, so the observations from the two techniques are not necessarily contradictory.

If the subunits of triose phosphate isomerase are identical, the observed isoenzymes cannot be due to random association of dissimilar subunits and may be conformational isoenzymes as previously suggested (Burton and Waley, 1968). Another possibility is that the multiple species are formed by conversion of specific glutamine or asparagine residues into glutamic acid or aspartic acid, respectively. In either case, the iso enzymes, particularly the minor ones, may be artifacts of isolation; indeed, the commercial preparations that we have examined by electrofocusing in polyacrylamide have a higher percentage of the minor components than do our preparations (Figure 5). This could partially account for the disturbing discrepancy between our findings and those of Lee and Snyder (1970); alternatively, their electrophoretic method may have higher resolving power than the one used in the present study. If one disregards the two minor species, the isoenzymic pattern observed by Lee and Snyder (1970) is similar to that anticipated on the basis of 2 chemically different subunits. Subtle differences in the two chains could have escaped detection by conventional methods. It will be of interest to subject the purified enzyme used in this study to Lee and Snyder's (1970) electrophoretic procedure when details become available. Elucidation of the precise nature of triose phosphate isomerase iso enzymes will require further study.

After this manuscript was submitted, a comparative study of rabbit muscle, rabbit liver, and yeast triose phosphate isomerase appeared (Krietsch *et al.*, 1970). The molecular weight and amino acid composition of the rabbit muscle enzyme reported by these investigators are in close agreement with the values reported here.

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