Energetics of Triosephosphate Isomerase: The Fate of the 1(*R*)-^{3}H Label of Tritiated Dihydroxyacetone Phosphate in the Isomerase Reaction†

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ABSTRACT: The isomerization of specifically tritium-labeled [1(*R*)-^{3}H]dihydroxyacetone phosphate to D-glyceraldehyde 3-phosphate, catalyzed by the enzyme triosephosphate isomerase, has been studied. The distribution of the ^{3}H label amongst the three possible sites (in [1(*R*)-^{3}H]dihydroxyacetone phosphate, in D-[2-^{3}H]glyceraldehyde 3-phosphate, and in the solvent) has been followed as a function of the extent of the reaction. It is shown that the extent of transfer of the ^{3}H label from the substrate dihydroxyacetone phosphate to D-glyceraldehyde 3-phosphate is between 3 and 6% (depending upon the extent of the reaction). The enzymic base responsible for proton abstraction from substrate is, therefore, in almost complete isotopic equilibrium with the solvent. The remaining substrate after partial reaction increases in specific radioactivity as the reaction proceeds, showing that the preferential reaction of ^{1}H substrate is more important than the washing out of ^{3}H label at the stage of the exchanging intermediate. Quantitatively, these results provide the data for the first steps in the analysis described in the previous paper (Albery, W. J., and Knowles, J. R. (1976), *Biochemistry*, preceding paper in this issue).

Triosephosphate isomerase catalyzes the interconversion of the two triose phosphates, dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. The crystalline enzyme from chicken muscle is a dimer of identical subunits each of molecular weight about 26 000 (Putman et al., 1972). It has no cofactor or specific metal ion requirements. As an isomerase, with a single substrate and a single product, it is particularly amenable to mechanistic study, and the equilibrium constant for the reaction is close enough to unity to allow the reaction to be run in either direction, utilizing the appropriate dehydrogenases as coupling enzymes (α-glycerophosphate dehydrogenase to remove dihydroxyacetone phosphate, or glyceraldehyde-phosphate dehydrogenase to remove glyceraldehyde phosphate). In the presence of excess of the appropriate dehydrogenase and cofactor, the reaction can be studied under conditions such that the isomerase step is rate limiting (Plaut and Knowles, 1972).

Aside from these attractive features, this system presents a rare opportunity for crystallographic and spectroscopic study. With most enzymes that use two or more substrates in either direction, even if one of these is water, it is currently impossible to study productive enzyme-substrate complexes directly by crystallographic methods. approaches to the problem of the structure of enzyme-substrate complexes have had to rely on the structures of enzyme-inhibitor complexes, or "dead-end" binary complexes or abortive ternary complexes (for two substrate systems). From such data, one has to try to deduce the structure of enzyme with real substrate bound (see, e.g., Blake et al., 1967; Steitz et al., 1969). For an isomerase, however, it is possible to find the structure both of native enzyme *and* of an enzyme-substrate complex. Progress with each of these problems is being made (Banner et al., 1971, 1975), and independent diffraction maps are being determined. An essential prerequisite for the complete analysis of the structural results in mechanistic terms is that both the course and the energetics of the catalyzed reaction be known. This is the object of the present work.

The early work of Rieder and Rose (1956, 1959) and of Bloom and Topper (1956, 1958) established the following characteristics of the reaction catalyzed by triosephosphate isomerase.

(a) If dihydroxyacetone phosphate is incubated with isomerase in tritiated water, about 1 g-atom of carbon-bound ^{3}H is incorporated per mol of resulting trioses. [The equilibrium mixture contains 96% of dihydroxyacetone phosphate and 4% of glyceraldehyde phosphate (Veech et al., 1969)]. The ^{3}H is stereospecifically incorporated on the C-1 carbinol carbon of dihydroxyacetone phosphate in the *pro-R* position. No significant labeling occurs in the absence of enzyme. Aldolase labilizes and exchanges the enantiomeric hydrogen on C-1, and the ^{3}H in aldolase-labeled dihydroxyacetone phosphate cannot be exchanged with the solvent by isomerase.

(b) When [^{3}H]dihydroxyacetone phosphate, specifically monotritiated by isomerase, is allowed to react with isomerase in untritiated water under "irreversible" conditions with the isomerase-catalyzed reaction rate limiting (i.e., in the presence of excess of glyceraldehyde-phosphate dehydrogenase, NAD^+,[1] and arsenate: this converts all free glyceraldehyde phosphate irreversibly to 3-phosphoglycerate), essentially all the ^{3}H of the substrate is lost to the medium.

(c) If an experiment similar to b is carried out with [^{3}H]dihydroxyacetone phosphate specifically labeled by aldolase, essentially all the ^{3}H is found in the NAD^3H, to which it is transferred from the aldehyde group of glyceraldehyde phosphate in the oxidation to phosphoglycerate. These experiments are summarized in Scheme I.

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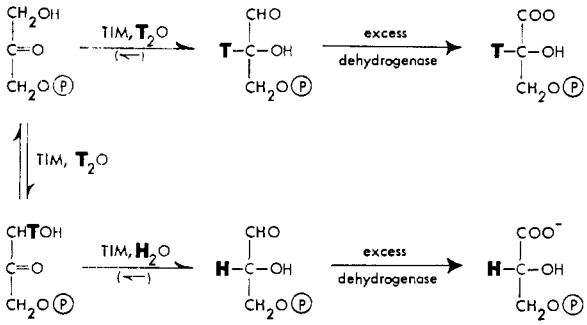
[1] Abbreviations used: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; DEAE, diethylaminoethyl; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid.

SCHEME I: Labeling of Substrate and of Product in the Isomerase-Catalyzed Reaction.[a]



[a] Top: Incorporation of ^3H into product during the isomerase-catalyzed reaction in tritiated water under irreversible conditions. Bottom: Loss of ^3H from specifically labeled dihydroxyacetone phosphate during the isomerase-catalyzed reaction under irreversible conditions. Vertical equilibrium: Stereospecific exchange of the *pro-R* hydrogen on C-1 of dihydroxyacetone phosphate at equilibrium, established by isomerase.

It is evident from these results that in the reaction catalyzed by triosephosphate isomerase, the hydrogen of the newly formed carbon-hydrogen bond is largely derived from the solvent, and we therefore have a situation in which neither substrate nor product can alone exchange carbon-bound hydrogen with the solvent, but an enzyme-bound intermediate can. This allows two kinds of experiment. First the fate of substrate hydrogen can be followed by running the reaction of specifically tritiated substrate in unlabeled water, and secondly the appearance in the product and in the remaining substrate of ^3H from tritiated water may be observed. The information gained from tracing the course of substrate hydrogens does not, as we shall see, duplicate that obtained from following solvent hydrogens, and provides additional information that is required for a complete solution of the energetic problem (Albery and Knowles, 1976a).

This paper describes the fate of ^3H in specifically labeled [1(*R*)-^3H]dihydroxyacetone phosphate, during the course of the enzyme-catalyzed isomerization of this substrate. There are three possible places where this ^3H label may be at any particular stage of the reaction: on the 1(*R*) position of remaining dihydroxyacetone phosphate, on the 2 position of the final product of the reaction, 3-phosphoglycerate, or in the solvent. Isotope in the solvent is not of interest here since all ^3H that is not bound to substrate or product carbon atoms will be in the solvent (or in equilibrium with it). Isotope on C-2 of the final product 3-phosphoglycerate is of especial interest, in that any label in this position will have been on C-2 of the product of the isomerase reaction, glyceraldehyde 3-phosphate. Thus any ^3H label in the 2 position of 3-phosphoglycerate represents a *transfer* of ^3H from C-1 of dihydroxyacetone phosphate to C-2 of glyceraldehyde phosphate. Finally, the ^3H content of the remaining substrate after partial reaction is of importance since this is determined both by any primary isotope effect leading to preferential consumption of 1(*R*)-^1H substrate rather than 1(*R*)-^3H material, and by the "washing out" effect, if any, due to an effective preequilibration of substrate with solvent via the enzyme-bound intermediate mentioned above. Changes in the specific radioactivity of the remaining labeled substrate during the course of the reaction will also affect the measured extent of label transfer from C-1 of dihydroxyacetone phosphate to C-2 of glyceraldehyde 3-phosphate.

The existence of any direct transfer of isotopic label between carbon centers in the triose phosphates has important implications in relation to both the number of enzyme catalytic groups involved in the proton transfers, and to the nature of these groups (Rose, 1962; Plaut and Knowles, 1972). Further, the variation in specific radioactivity of starting material during the course of the isomerization process provides additional information on the energetics of the enzyme-catalyzed reaction (Albery and Knowles, 1976a).

Experimental Section

Materials. Enzymes. Triosephosphate isomerase was prepared by J. Law Esq. according to the method of Putman et al. (1972). A value of $E_{280}^{0.1\%}$ of 1.21 was assumed. 3-Phosphoglycerate kinase (from yeast, as a crystalline suspension in ammonium sulfate), D-glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle, as a crystalline suspension in ammonium sulfate), aldolase (from rabbit muscle, as a crystalline suspension in ammonium sulfate), and phosphoglyceromutase (from rabbit muscle, as a crystalline suspension in ammonium sulfate) were obtained from the Sigma Chemical Co. (London, England). Enolase (from rabbit muscle, as a crystalline suspension in ammonium sulfate) and glycerophosphate dehydrogenase (from rabbit muscle, as a suspension in ammonium sulfate) were obtained from Boehringer Mannheim Ltd. (London, England). Before using either of the dehydrogenases, any contamination by isomerase was removed by chlorohydroxyacetone phosphate treatment (see de la Mare et al., 1972) and the ammonium sulfate removed by dialysis overnight.

Cofactors. NAD^+, NADH (disodium salt), and ATP (disodium salt) were obtained from the Sigma Chemical Co.

Dihydroxyacetone phosphate (as the dicyclohexylammonium salt of the dimethyl ketal), fructose 1,6-diphosphate (tetrasodium salt), 2,3-diphosphoglycerate (pentacyclohexylammonium salt), Dowex 50W (H^+ form, 100–200 mesh, 4% cross-linked) and Dowex 1 (Cl^- form, 200–400 mesh, 8% cross-linked) were obtained from the Sigma Chemical Co. DEAE-cellulose (DE 52) was obtained from Reeve Angel Co., Ltd. (London, England). Tritiated water (5 Ci/ml) was obtained from The Radiochemical Center (Amersham, England). All other materials were obtained from British Drug Houses, Ltd., and were the highest grade available. Buffer solutions were prepared with the highest grade components, using deionized water.

[*1*(*R*)-^3H]*Dihydroxyacetone Phosphate.* Dihydroxyacetone phosphate (20 μmol in 1.0 ml) was added to 1 M triethanolamine-HCl buffer, pH 7.4 (0.2 ml), in the presence of ^3H_2O (15 μl of a solution of 5 Ci/ml). Triosephosphate isomerase (25 μl of a solution of 17 mg/ml) was added and the mixture left at room temperature for 90 min. After this time the mixture was cooled to 0 °C and the pH was taken to 4 by adding 1 M HCl. This solution was applied to a column (12 cm × 1.77 cm^2) of Dowex 1 (Cl^-), equilibrated with 0.16 mM HCl, pH 3.8. Excess of tritiated water was removed by washing the column with 0.16 mM HCl, until the radioactivity of the eluate was close to background, and the mixture of tritiated triose phosphates ([1(*R*)-^3H]dihydroxyacetone phosphate, 96%, and D-[2-^3H]glyceraldehyde 3-phosphate, 4%) was eluted with a nonlinear pH gradient (80 + 80 ml; 0.16 mM HCl to 0.1 M HCl), 1-ml fractions being collected. Fractions containing dihydroxyacetone phosphate were pooled (ca. 25 ml) and, in most experiments, treated with isomerase-free aldolase (de la Mare et al., 1972) to remove the radioactive glyceraldehyde phosphate. The pooled fractions were concentrated to small volume (ca. 4 ml) by freeze-drying, and neutralized with 1 M NaOH to pH 7.5. Aldolase (50 μl of a solution of 10 mg/ml) was then added and the mixture left at room tem-

perature for 1 h. The reaction mixture was cooled to 0 °C and the pH taken to 4 by adding 1 M HCl. This solution (containing [1(*R*)-^{3}H]dihydroxyacetone phosphate and tritiated fructose 1,6-diphosphate) was applied to a column (12 cm × 1.77 cm^2) of Dowex 1 (Cl^-), equilibrated with 0.16 mM HCl, pH 3.8. The column was washed with 0.16 mM HCl (ca. 20 ml), and the phosphate esters were eluted with a nonlinear pH gradient as above. Under these conditions, dihydroxyacetone phosphate is completely separated from fructose 1,6-diphosphate. The fractions containing dihydroxyacetone phosphate (ca. 25 ml) were freeze-dried, the residue was dissolved in water, and the pH was adjusted to 4 for storage at −20 °C. The specific radioactivity of the [1(*R*)-^{3}H]dihydroxyacetone phosphate prepared by this method was in the region of 3 × 10^5 cpm/μmol.

Methods. Scintillation counting was done with a Beckman DPM-100 automatic liquid scintillation counter. The scintillation fluid, containing A.R. toluene (350 ml), ethanol (160 ml), naphthalene (30 g), 2,5-diphenyloxazole (1.5 g), and 1,4-di[2-(5-phenyloxazolyl)]benzene (60 mg), was stored in the dark. Samples (50 μl) for specific radioactivity determinations were added to 6 ml of scintillation fluid. Background counts were determined on each vial. pH measurements were made on a Radiometer TT1C instrument fitted with a pHA 630 scale expander, standardized against British Drug Houses Ltd. standard buffer solutions. Conductivity was determined with a Radiometer CDM 2e instrument. Ultraviolet absorbance measurements were made with a Unicam SP.1800 spectrophotometer. An extinction coefficient for NADH of 6220 M^{-1} cm at 340 nm was assumed (Horecker and Kornberg, 1948).

Isomerase-Catalyzed Reaction. In order to determine accurately the small amount of glyceraldehyde phosphate in the preparation of tritiated dihydroxyacetone phosphate, the actual isomerase-catalyzed reaction was run as follows. Into a 10-mm light-path optical cuvette were placed 100 mM triethanolamine-HCl buffer (pH 7.6), EDTA (5 mM), sodium arsenate (3 mM), NAD^+ (2 to 4 mM), and [1(*R*)-^{3}H]dihydroxyacetone phosphate (0.7 to 2.0 mM). The volume was 2.80 ml. After temperature equilibration to 30 °C, the absorbance at 340 nm was measured and isomerase-free glyceraldehyde-phosphate dehydrogenase (de la Mare et al., 1972) (25 μl of a solution of 5 mg/ml) was added. The absorbance change at 340 nm was a measure of the glyceraldehyde phosphate content of the solution (though the change was corrected for any dehydrogenase absorption or light scattering by subtracting the absorption change on the addition of a second sample (25 μl) of the dehydrogenase solution). The order of reagent addition provided an upper limit on any contamination by glyceraldehyde phosphate. This method is capable of estimating >0.05% of contamination of the substrate by glyceraldehyde phosphate. After the addition of a further sample (250 μl) of glyceraldehyde-phosphate dehydrogenase, the isomerization reaction was initiated by the addition of triosephosphate isomerase (25 μl of a solution of 10 μg/ml). The extent of reaction was monitored by the absorbance at 340 nm or 366 nm of a sample of the reaction mixture in a 2-mm light-path optical cuvette. The reaction in the larger (10 mm) cuvette was stopped at the desired point by lowering the pH to below 4 by the addition of 1 M HCl (120 μl) and rapid cooling to 0 °C (initially in an acetone–solid CO_2 bath). Isomerase is completely inactive under these conditions (Plaut and Knowles, 1972). The reaction in the smaller (2 mm) cuvette was allowed to proceed to completion.

From the quenched reaction mixture, the product and the remaining substrate were separated by a procedure based on that of Maister et al. (1976) but employing a lower pH to minimize hydrolysis of dihydroxyacetone phosphate. The quenched reaction mixture was passed through a small column (2 cm × 1.7 cm^2) of Dowex 50 (H^+) at pH 3.8 to remove the enzymes before the pH of the eluate (ca. 15 ml) was raised to 7.1 with ammonium hydroxide. This solution was diluted with deionized water to about 80 ml (conductivity <400 μ*S*) and applied to a column (15 cm × 2 cm^2) of DEAE-cellulose (DE 52), equilibrated at 4 °C with 5 mM triethanolamine-HCl buffer, pH 7.1. A linear gradient (160 ml) of triethanolamine-HCl (5–300 mM) was applied to the column, and fractions of about 1 ml were collected. The pH of each fraction was lowered by the addition of 2 drops of 1 M HCl immediately after collection. Under these conditions the yield of 3-phosphoglycerate was 100% and the yield of dihydroxyacetone phosphate was at least 80%.

Determination of Specific Radioactivity. The specific radioactivity of dihydroxyacetone phosphate (both the 1(*R*)-^{3}H starting material and that recovered from the reaction mixture after partial reaction) was determined as described by Maister et al. (1976). In order that the specific radioactivity of dihydroxyacetone phosphate recovered from the reaction mixture could be directly compared with the specific radioactivity of starting material under the same conditions of quenching, a blank incubation was carried out. This contained 100 mM triethanolamine-HCl buffer, pH 7.6, EDTA (5 mM), sodium arsenate (3 mM), NAD^+ (2 mM) and [1(*R*)-^{3}H]dihydroxyacetone phosphate (0.7 mM) in a volume of 1 ml. No enzymes were added to this mixture, it was otherwise treated in exactly the same way as the reaction mixture, and the dihydroxyacetone phosphate was isolated from an identical DEAE-cellulose column.

Radioactivity determinations were normally made directly on duplicate samples (50 μl) of the column eluate.

The specific radioactivity of the reaction product, 3-phosphoglycerate, was determined as described by Maister et al. (1976), but a further check was required to find what proportion of the tritium label was on the 2 position. After any volatile radioactivity had been removed from the 3-phosphoglycerate fractions by the distillation technique outlined by Maister et al. (1976), the solid residue was dissolved in deionized water (5 ml), the pH was raised to 7.4 with 1 M NaOH (25 μl), and $MgCl_2$ (150 μl of a 500 mM solution), 2,3-diphosphoglycerate (25 μl of a 10 mM solution), phosphoglyceromutase (25 μl of a 10 mg/ml solution), and enolase (50 μl of a 25 mg/ml solution) were added. The mixture was left at 25 °C for 1 h, after which the reaction was stopped by the addition of 1 M HCl (20 μl). The water was now distilled and subjected to scintillation counting. Control experiments were performed with genuine samples of 3-phospho[2-^{3}H]glycerate, prepared as follows. Dihydroxyacetone phosphate (0.9 mM) was incubated at room temperature in triethanolamine-HCl buffer (pH 7.4) (120 mM), EDTA (36 mM), NAD^+ (1.4 mM), sodium arsenate (4.5 mM), glyceraldehyde-phosphate dehydrogenase (50 μl of a solution of 10 mg/ml), triosephosphate isomerase (25 μl of a solution of 17 mg/ml), and ^3H_2O (1.7 mCi/ml). The total volume was 4.44 ml. The course of the reaction was followed by monitoring a sample in a 2-mm light-path optical cuvette, and the reaction was allowed to go to completion. The reaction mixture was then cooled to 0 °C and the pH lowered to 4. Chromatography on a column (5 cm × 0.75 cm^2) of Dowex 1 (Cl^-), as used in the preparation of [1(*R*)-^{3}H]dihydroxyacetone phosphate (see above), yielded 3-phospho[2-^{3}H]glycerate of approximate

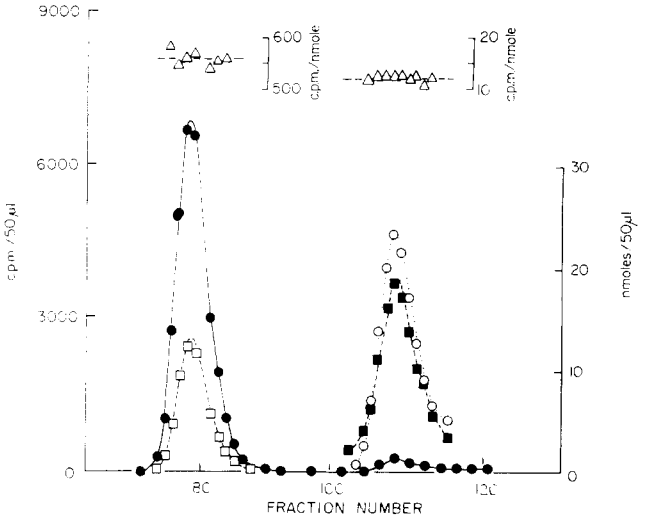


FIGURE 1: Separation of dihydroxyacetone phosphate and 3-phosphoglycerate on DEAE-cellulose. The column was eluted with a linear gradient of triethanolamine-HCl (5–300 mM), pH 7.1, 4 °C. The sample was the quenched reaction mixture (after removal of isomerase) after 63% conversion of dihydroxyacetone phosphate into 3-phosphoglycerate. (- -□- -) Micromoles of dihydroxyacetone phosphate per fraction; (- -■- -) μmol of 3-phosphoglycerate per fraction; (—●—) cpm in 50 μl; (···○···) cpm in 1 ml; (—△—) specific radioactivity.

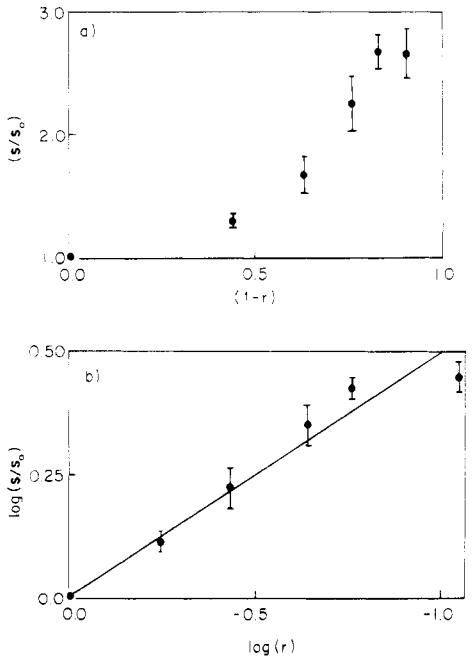


FIGURE 2: (a) Variation in specific radioactivity of the substrate [1(*R*)-^{3}H]dihydroxyacetone phosphate (**s**) with the fractional extent of reaction ($1 - r$). At the start of the reaction, $r = 1$ and $\mathbf{s} = \mathbf{s}_0$. (b) Plot of the data of Figure 2a according to eq 1. r is the fraction of substrate remaining.

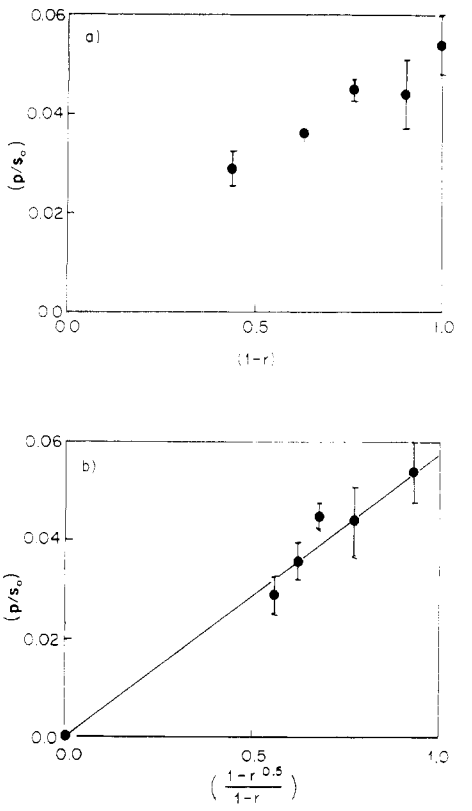


FIGURE 3: (a) Variation in specific radioactivity of the product 3-phospho[2-^{3}H]glycerate (**p**) with the fractional extent of reaction ($1 - r$). At the start of the reaction, $r = 1$ and $\mathbf{s} = \mathbf{s}_0$. (b) Plot of the data of Figure 3a according to eq 2. r is the fraction of substrate remaining, and A_6' is derived from Figure 2b (see the text).

specific radioactivity 2.5×10^4 cpm/μmol. This material was subjected to the "washing out" procedure by incubation with phosphoglyceromutase and enolase as described above. After incubation for 1 h, essentially all the radioactive label appeared in the distilled water. In the absence of enzymes, no radioactive label was removed from the 3-phospho[2-^{3}H]glycerate.

Results

Two sets of experiments were run, one in which the specific radioactivity of remaining substrate, dihydroxyacetone phosphate, was determined, and one in which the specific radioactivity of the product 3-phosphoglycerate was established.

After the reaction was stopped and the isomerase removed, the remaining substrate and the product were isolated chromatographically. Column elution profiles obtained in typical experiments are shown in Figure 1.

Experiments were performed at six different extents of reaction. The variation in specific radioactivity of recovered starting material, **s** (expressed as a fraction of the specific radioactivity at the start of the reaction, $\mathbf{s}/\mathbf{s}_0$) with the extent of the reaction, ($1 - r$), is shown in Figure 2a. The extent of tritium transfer from [1(*R*)-^{3}H]dihydroxyacetone phosphate to [2-^{3}H]glyceraldehyde 3-phosphate (expressed as a fraction of the specific radioactivity of the starting material, $\mathbf{p}/\mathbf{s}_0$) is plotted against the extent of the reaction, ($1 - r$), in Figure 3a.

In order to check on the location of the ^{3}H label in the product 3-phosphoglycerate, this material was treated with phosphoglycerate mutase and enolase, which specifically washes out hydrogen on C-2 into the solvent (Dinovo and Boyer, 1971). The solvent was separated from the involatile salts (which contain 3-phosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate) by freeze-drying in a closed system. The remaining solid was dissolved in deionized water and samples were taken for scintillation counting from this solution and from the solvent distillate. Results for 3-phosphoglycerate obtained by isomerase-catalyzed reaction of [1(*R*)-^{3}H]dihydroxyacetone phosphate and for an authentic sample of 3-phospho[2-^{3}H]glycerate are shown in Table I. It is evident that 90% or more of the radioactivity in both the experimental and

TABLE I: Demonstration of the Position of the ^3H Label in 3-Phospho-D-glycerate.

	3-Phosphoglycerate[a]	
Total cpm in 3-phosphoglycerate	14 000	17 100
Total cpm washed out from C-2 by mutase and enolase[b]	16 500	17 700
Total cpm remaining (nonvolatile counts)	400	2 000

	3-Phospho[2-^3H]glycerate, authentic[c]			
	Low Salt[c]		High Salt[d]	
Total cpm in 3-phosphoglycerate	11 400	25 710	12 370	12 370[e]
Incubation time with mutase and enolase (min)	40	300	60	60[e]
Total cpm washed out from C-2[b]	9 580	24 420	13 200	0[e,f]

[a] Duplicate experiments from [1(*R*)-^3H]dihydroxyacetone phosphate after conversion catalyzed by triosephosphate isomerase. [b] For details, see Experimental Section. [c] From 3-phosphoglycerate (0.5 mM) (prepared from dihydroxyacetone phosphate after equilibration with isomerase in tritiated water) in 50 mM triethanolamine-HCl buffer, pH 7.4, at 25 °C for the times stated. [d] 2 M in KCl. [e] In the absence of mutase and enolase. [f] Within 10% of background.

control samples of 3-phosphoglycerate is washed out into the solvent in the presence of phosphoglycerate mutase and enolase. Differential quenching in the samples causes some uncertainty in the values but an upper limit of 10% can be set for the existence of adventitious tritium on C-3 rather than on C-2. That is, percentage extents of transfer of tritium from [1(*R*)-^3H]dihydroxyacetone phosphate to 3-phospho[2-^3H]glycerate (via the isomerase product [2-^3H]glyceraldehyde 3-phosphate) may have been overestimated by no more than 0.3 to 0.6%.

Discussion

Tritium Transfer. Two reports in the literature have presented evidence concerning the transfer of tritium between carbon centers in the triose phosphates during the isomerase-catalyzed reaction. Rieder and Rose (1959), using specifically tritiated dihydroxyacetone phosphate, suggested that there was no transfer of the 1(*R*)-^3H label to the 2 position of glyceraldehyde phosphate. However, the aim of this work was to eliminate a hydride shift pathway for the isomerase-catalyzed reaction (for which complete transfer with no loss of ^3H to the solvent would have been expected), and dihydroxyacetone phosphate of relatively low specific radioactivity was used. It is doubtful if less than 10% of transfer would have been detected. Simon and his collaborators (Simon et al., 1968), using D-[3-^3H]glucose, hexokinase, phosphoglucose isomerase, phosphofructokinase, and aldolase to prepare specifically labeled dihydroxyacetone phosphate in situ, reported that some 10% of the ^3H in dihydroxyacetone phosphate was transferred intramolecularly to glyceraldehyde phosphate in the isomerase-catalyzed reaction. The validity of this experiment depends, inter alia, on the isotopic purity of the tritiated glucose, and Dr. Simon has recently informed us that because some 5% of the ^3H label on the starting glucose was in the 6 position, the revised estimate of the extent of tritium transfer (from dihydroxyacetone phosphate to glyceraldehyde phosphate) in the yeast isomerase-catalyzed reaction is about 5% at pH 8.7. The value may be lower than this for the reaction at pH 7.6 (Dr. H. Simon, private communication).

In the light of the above, it is evident that the percentage of the ^3H label that is transferred directly from C-1 of dihydroxyacetone phosphate to C-2 of glyceraldehyde phosphate may be very small. Possible sources of error must therefore be considered carefully, even if (as in the present experiments) substrate of very high specific radioactivity is used.

A value for the percentage of ^3H transfer that is too high would be obtained (a) if the [1(*R*)-^3H]dihydroxyacetone phosphate substrate is contaminated with [^3H]glyceraldehyde phosphate, (b) if there is any ^3H label attached to C-3 of the substrate, or (c) if the 3-phosphoglycerate product is contaminated with any other radioactive material. Considering point a, any [^3H]glyceraldehyde phosphate will be converted by the coupling dehydrogenase into 3-phosphoglycerate of the same specific radioactivity. Thus contamination of the substrate by only 1% of [^3H]glyceraldehyde phosphate will give an apparent transfer of 1% for a reaction that has run to completion, or 2% for a reaction quenched after half the dihydroxyacetone phosphate has been consumed. In the method of preparation of the [^3H]triose phosphates, the 4% of [^3H]glyceraldehyde phosphate present at equilibrium (Veech et al., 1969) was reduced by omitting from the pool early fractions that contain higher concentrations of glyceraldehyde phosphate. In some experiments, the remaining 2% or so of [^3H]glyceraldehyde phosphate was removed from the [1(*R*)-^3H]dihydroxyacetone phosphate by incubation with isomerase-free aldolase (de la Mare et al., 1972), the equilibrium constant for which is 10^4 M in favor of fructose 1,6-diphosphate (Rutter, 1961). These precautions were exercised in two experiments (at 63 and 100% conversion of substrate to product), the results from these runs being in good agreement with those from less elaborate experiments.

Immediately before the isomerase-catalyzed reaction of dihydroxyacetone phosphate, the substrate was assayed for traces of glyceraldehyde phosphate, so that the measured specific radioactivity of the product 3-phosphoglycerate could be corrected for any such contaminating traces. Considering point b, if, during the tritiation of triose phosphates by isomerase, any ^3H is incorporated nonenzymically into C-3, this may remain bound and be counted in the product 3-phosphoglycerate. This problem, and that under point c (above), can be solved by the direct determination only of ^3H label on C-2 of 3-phosphoglycerate, by the "washing out" procedure using phosphoglycerate mutase and enolase.

A value of the percentage of ^3H transfer that is too low would be obtained (d) if some of the ^3H in the reaction mixture at the start is not bound to the dihydroxyacetone phosphate at all, or (e) if some of the ^3H in dihydroxyacetone phosphate is bound to the 1(*S*) position and is then lost in the reaction to 3-phosphoglycerate. Finally, (f) if the isomerase reaction is not irreversible, then equilibration of product glyceraldehyde phosphate back to dihydroxyacetone phosphate will remove any ^3H label on C-2 of the glyceraldehyde phosphate into the medium. Point d is unlikely, since the material used as substrate has been subjected to three ion-exchange chromatographic purification steps during its preparation. Point e is taken care of by the fact that any label in the 1(*S*) position will be completely removed by the aldolase incubation (in ^1H_2O) used to rid the substrate of glyceraldehyde phosphate. Point f, the question of irreversibility, was checked in the present work by ensuring that an even greater excess of the coupling enzyme glyceraldehyde-phosphate dehydrogenase had no effect upon the rate of the isomerase-catalyzed reaction. That the conditions used here are "safe" is also evident from the results of Plaut and Knowles (1972). Lastly, a potential source of error that could affect the observed extent of transfer either way is the varying efficiency of scintillation counting of sam-

ples of differing pH and ionic strength. This error was eliminated by measuring the specific radioactivity of unreacted dihydroxyacetone phosphate, of dihydroxyacetone phosphate recovered from the reaction mixture, and of 3-phosphoglycerate, under identical conditions.

On the basis of the foregoing, we consider that a proper estimate of the extent of tritium transfer in the isomerase-catalyzed reaction has been made.

The existence of a small extent of direct transfer of tritium between carbon centers has a number of important implications. First, the analogy between triosephosphate isomerase and phosphoglucose isomerase is more complete. On the basis of some 50% of transfer in the phosphoglucose isomerase reaction, Rose (1962) proposed that a single-base mechanism was most probable, and that (assuming that a single base would act only on one side of the substrate for the shuttling of protons) the stereochemistry of the aldose and the labilized hydrogen of the corresponding ketose required a *cis*-enediol intermediate. The same reasoning can now be applied to triosephosphate isomerase, provided (a) that a single mechanistic pathway operates and (b) that the proton abstraction and readdition processes do occur on the same side of the substrate. Secondly, the existence of only about 3–6% of transfer shows that the enzymic base responsible is relatively accessible to solvent and is in almost complete isotopic equilibrium with the solvent. Thirdly, the knowledge of the partitioning of the enediol species (structure B of Scheme II of Maister et al., 1976), which exchanges (to A) some 20 to 30 times more readily than it transfers tritium (to D) to give product, provides information about the overall proton exchange rate of the conjugate acid of the enzyme base. The possible limitations on the pK_a of this conjugate acid have been discussed (Plaut and Knowles, 1972).

Specific Radioactivity of Remaining Substrate. The second piece of mechanistic information obtained from these experiments is the variation in the specific radioactivity of the substrate after partial reaction. This quantity is determined both by the primary isotope effect, which results in the consumption of protonated dihydroxyacetone phosphate in preference to the tritiated material, and by the partitioning ratio of the enediol intermediate on which depends the rate at which the tritium label is "washed out" by preequilibration of the substrate. There is, therefore, a balance between effects here, the isotope effect tending to increase the specific radioactivity of the remaining substrate, and the "washing out" effect tending to decrease it. From Figure 2a it is evident that the specific radioactivity of the remaining reactant is some three times higher near the end of the reaction than at the beginning. This increase in the radioactivity of the starting material is the cause of the parallel rise in specific radioactivity of the product as the reaction proceeds (see Figure 3a).

Quantitative Treatment. In terms of the full theoretical treatment already presented (Albery and Knowles, 1976a), the study of the specific radioactivity of the remaining substrate is a TS′**s** experiment (T, tritium; S′, isotope starts in the substrate dihydroxyacetone phosphate; **s**, analyze for isotope in the substrate remaining after partial reaction). From Table III of Albery and Knowles (1976a), the equation for this type of experiment is:

$$\ln (\mathbf{s}/\mathbf{s}_0) = (A_6' - 1) \ln r \qquad (1)$$

[where **s** is the specific radioactivity of the dihydroxyacetone phosphate when a fraction r of it remains (at $r = 1$, $\mathbf{s} = \mathbf{s}_0$)]. In Figure 2a is plotted the experimental variation in specific radioactivity of recovered starting material (expressed as a fraction of the specific radioactivity at the start of the reaction), $\mathbf{s}/\mathbf{s}_0$, with the extent of the reaction, $(1 - r)$. In Figure 2b, these data are plotted according to eq 1 and yield a reasonable straight line through the origin, giving a value for A_6' of 0.50 ± 0.03 (the error quoted is the standard error).

The experiment in which the degree of tritium transfer is observed is a TS′**p** experiment (T, tritium; S′, isotope starts in the substrate dihydroxyacetone phosphate; **p**, analyze for isotope in the product). From Table III of Albery and Knowles (1976a), the relevant equation is:

$$\mathbf{p}/\mathbf{s}_0 = A_5'(1 - r^{A_6'})/(1 - r) \qquad (2)$$

where the specific radioactivity of the product, **p**, is expressed as a fraction of the original specific radioactivity of the starting material, $\mathbf{s}_0$. In Figure 3a is plotted the variation of $\mathbf{p}/\mathbf{s}_0$ with the extent of the reaction $(1 - r)$. In Figure 3b, these data are plotted according to eq 2, knowing A_6', and yield a reasonable straight line through the origin, giving a value for A_5' of 0.058 ± 0.004.

The linearity of the plot shown in Figure 3b both confirms the value of A_6' obtained from the TS′**s** experiment and also suggests that the possible systematic errors discussed above for the tritium transfer experiment are indeed negligible.

Further analysis of the parameters A_6' and A_5' derived from the experiments described in this paper is presented in Albery and Knowles (1976b).

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Energetics of Triosephosphate Isomerase: The Appearance of Solvent Tritium in Substrate Dihydroxyacetone Phosphate and in Product†

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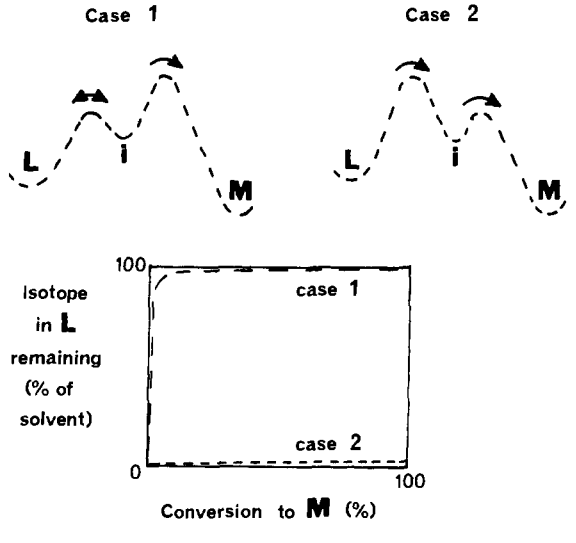
ABSTRACT: When the isomerization of dihydroxyacetone phosphate to D-glyceraldehyde 3-phosphate is catalyzed by triosephosphate isomerase in tritiated water, both the substrate and product become labeled. The specific radioactivity of the product is about 80% that of the solvent, which shows that the protonation of the enediol intermediate at C-2 (to form the enzyme-bound product D-glyceraldehyde 3-phosphate) is followed by a slower step not involving proton transfer. The specific radioactivity of the remaining substrate after partial reaction rises as the reaction proceeds and shows that the reaction intermediate that exchanges protons with the medium returns to dihydroxyacetone phosphate (picking up tritium) about one-third as often as it is converted to D-glyceraldehyde 3-phosphate. These results allow a qualitative description of the relative heights of the energy barriers in the catalyzed reaction and contribute to the quantitative analysis of the energetics of the process.

As is discussed in the previous paper (Herlihy et al., 1976), the fact that the pathway of the reaction catalyzed by triosephosphate isomerase involves an intermediate that can exchange protons with the solvent allows the study of some of the details of the catalysis by observing the fate of particular carbon-bound hydrogens during the enzyme-catalyzed reaction. For a more complete description of the energetics of the isomerase-catalyzed reaction, however, we must investigate not only the fate of substrate protons, but also the fate of solvent protons. Since we have for the triosephosphate isomerase catalyzed reaction a situation in which neither substrate nor product alone can exchange hydrogen with the solvent, but an enzyme-bound intermediate can, we can monitor the appearance of ^3H (from tritiated water) in the product and in the remaining substrate. Two types of experiment are possible. First, the discrimination between hydrogen isotopes in the collapse to product of the intermediate that equilibrates with solvent can be studied. This will provide information about the steps after the formation of the intermediate. Secondly, since only the intermediate can exchange with solvent, the partitioning of this species (returning to substrate on the one hand, and proceeding to product on the other) can be investigated. Thus, provided that the intermediate exchanges rapidly with solvent, we can distinguish between case 1 and case 2 (Scheme I). For a reaction L → M, with an intermediate i that can exchange, in case 1, the system will effectively preequilibrate and L will rapidly (at low L-to-M conversions) attain isotopic equilibrium with solvent. In case 2, where the intermediate i partitions very much in favor of M, little or no isotope will be incorporated into L, even after most of L has been converted into M. Product M will, of course, always contain isotope. The measured dependences of isotopic content of starting material, L, on the extent of conversion of L to M, will be as shown in Scheme I. Intermediate cases, in which the partitioning of the intermediate is less extreme, may provide enough information to determine the partition ratio quantitatively.

SCHEME I: Limiting Cases for the Incorporation of Isotope (Introduced Only by Exchange at i) into Starting Material L for a Reaction Performed under Irreversible Conditions in the Direction L → M.



In this paper we report both types of experiment: the isotopic discrimination in product formation, and the exchange vs. conversion study, for the isomerase-catalyzed reaction of dihydroxyacetone phosphate.

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