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Energetics of Triosephosphate Isomerase: The Appearance of Solvent Tritium in Substrate Glyceraldehyde 3-Phosphate and in Product†

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ABSTRACT: When the isomerization of D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate is catalyzed by triosephosphate isomerase in tritiated water, both the substrate and the product become labeled. The specific radioactivity of the product is only about 13% that of the solvent, which shows that the protonation of the enediol intermediate at C-1 (to form the enzyme-bound product dihydroxyacetone phosphate) is a kinetically significant step, and that the rate of loss of dihydroxyacetone phosphate from the enzyme is relatively fast. 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 D-glyceraldehyde 3-phosphate about one-third as often as it is converted to dihydroxyacetone phosphate. These results confirm the qualitative description of the relative heights of the energy barriers in this reaction and further contribute to the quantitative analysis of the free-energy profile.

In the previous paper (Maister et al., 1976) we have pointed out that, in the reaction catalyzed by triosephosphate isomerase, the hydrogen of the newly formed carbon–hydrogen bond is derived from the solvent and that, although neither substrate nor product can alone exchange hydrogen with the solvent, an enzyme-bound reaction intermediate can. This allowed two kinds of experiment to be performed. First, the collapse to product of the intermediate that is in isotopic equilibrium with solvent yields radioactively labeled product, the specific activity of which provides information about the product-forming step. Secondly, the partitioning of the intermediate between product and substrate can be studied by measuring the specific radioactivity of the substrate remaining after partial reaction. This experiment provides information about the energy barriers either side of the intermediate, that is, about the way in which the intermediate partitions between the two paths open to it (back to substrate, or on to product).

With dihydroxyacetone phosphate as substrate, we have seen (Maister et al., 1976) that the discrimination against tritium in the formation of the product (D-glyceraldehyde 3-phosphate) is 1.3-fold, which is very much smaller than that expected for a primary kinetic tritium isotope effect. This fact requires that a step in the reaction *after* the formation of the enzyme–glyceraldehyde phosphate complex be rate limiting. The likely situation is that the rate of loss of glyceraldehyde 3-phosphate from the enzyme–product complex is slower than the collapse of the intermediate to give that complex. The second type of experiment yielded the dependence of the tritium content of the remaining substrate dihydroxyacetone phosphate on the extent of the reaction. This dependence (see Figure 1 of Maister et al., 1976) provided both qualitative and quantitative information about the partitioning of the reaction intermediate.

Unlike many enzyme-catalyzed reactions, the overall equilibrium constant of the transformation catalyzed by triosephosphate isomerase allows the reaction to be studied in either direction. In the present paper we report the results of the two experiments described above, namely isotope discrimination in product formation, and exchange vs. conversion, for the isomerase-catalyzed reaction with D-glyceraldehyde 3-phosphate as substrate. It will be apparent that new information about the catalysis is forthcoming, from the fortunate possibility of being able to study the "back" reaction as well as the "forward" reaction. Also reported here are the *equilibrium* isotope fractionation factors for the two substrates in equilibrium with tritiated water.

Experimental Section

Materials. Enzymes, cofactors, substrates, and other materials were as described by Herlihy et al. (1976). DL-Glyceraldehyde 3-phosphate was obtained from the Sigma Chemical Co. (London, England) either as the barium salt of the diethyl acetal or as an aqueous solution of the liberated material.

Methods. Measurements of radioactivity, pH, conductivity, and ultraviolet absorbance were done as described by Herlihy et al. (1976).

Isomerase-Catalyzed Reactions. The transformation of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate catalyzed by triosephosphate isomerase was coupled to the reduction of dihydroxyacetone phosphate by α-glycerophos-

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phate dehydrogenase with NADH.[1] The reactions were performed in a 10-mm light-path optical cuvette, in a total volume of 2.365 ml, containing: triethanolamine-HCl buffer, pH 7.6 (100 mM), EDTA (20.8 mM), NADH (0.96 mM), D-glyceraldehyde 3-phosphate (0.45 mM), glycerophosphate dehydrogenase (0.38 mg/ml), and tritiated water (96 mCi/ml), and the reaction was initiated with triosephosphate isomerase (0.192 μg/ml). All the solutions (except isomerase) were preequilibrated at 30 °C in the cuvette. Before addition of isomerase, two samples (10 μl) were withdrawn for determination of the specific radioactivity of the solution. After the addition of isomerase the course of the reaction was followed by monitoring the disappearance of NADH at 366 nm. This wavelength (rather than the λ_{max} at 340 nm) was used so that the initial absorbance was less than 2. An extinction coefficient for NADH at 366 nm of 3300 M^{-1} cm was used (Hohorst, 1956). The reaction was stopped after the desired extent of reaction had been reached, by lowering the pH to about 3.5 by the addition of 1 M HCl (210 μl) and rapid cooling to 0 °C. Isomerase is completely inactive under these conditions (Plaut and Knowles, 1972).

Separation Methods. Before separating the components of the reaction mixture, the solvent was removed by freeze-drying in order to reduce the background level of radioactivity in the column eluate and to minimize the amount of column washing required to reduce the eluting radioactivity to acceptable levels. The quenched reaction mixture was freeze-dried in vacuo as described by Herlihy et al. (1976). The residue was dissolved in 0.16 mM HCl and applied to a column (10 cm × 0.76 cm^2) of Dowex 1 (8% cross-linked, 200–400 mesh) that had been converted to the bisulfite form by treatment with saturated sodium bisulfite (100 ml), followed by water (250 ml) and 0.16 mM HCl (250 ml). After application of the reaction mixture, the column was washed with 0.16 mM HCl until the radioactivity of the eluate reached background levels. A nonlinear gradient elution of 0.16 mM HCl (40 ml) to 1.0 M HCl (20 ml) resulted in the separation of glyceraldehyde 3-phosphate and *sn*-glycerol 3-phosphate. Fractions of ca. 1 ml were collected.

Determination of the Tritium Fractionation Factors for Dihydroxyacetone Phosphate (Φ_S) and Glyceraldehyde 3-Phosphate (Φ_P). Dihydroxyacetone phosphate (20 mM) was incubated in triethanolamine-HCl buffer, pH 7.4 (100 mM), at 30 °C for 2 h in the presence of triosephosphate isomerase (12.5 μg/ml) and tritiated water (10 μl/ml of 5 Ci/ml). Duplicate samples (25 μl) were taken before and after the incubation and diluted into deionized water (500 ml) from which portions (50 μl) were taken for scintillation counting. After the incubation, 1 M HCl (150 μl) was added and the mixture was frozen and freeze-dried in a closed system (see Herlihy et al., 1976) to remove most of the tritiated water. The residue was dissolved in water (5 ml) and passed through a column (3.0 cm × 0.4 cm^2) of Dowex 50 (H^+ form) to remove isomerase.

In experiments where the fractionation factors for the separated triose phosphates were measured (rather than the composite fractionation factor for the 96% of dihydroxyacetone phosphate + 4% glyceraldehyde 3-phosphate), the glyceraldehyde 3-phosphate was converted enzymically to 3-phosphoglycerate to facilitate separation from dihydroxyacetone phosphate. The pH of the eluate containing the triose phosphates was raised to ca. 7 with aqueous ammonia, and the following reagents were added: triethanolamine-HCl buffer, pH 7.4 (200 mM; 2 ml), containing EDTA (10 mM), sodium arsenate (10 mM), NAD^+ (7 mg), and isomerase-free glyceraldehyde-phosphate dehydrogenase (500 μl of a solution containing 0.5 mg/ml). When the absorbance at 340 nm had reached a constant value, the pH of the mixture was adjusted to 4 with 1 M HCl and the solution passed through a column of Dowex 50 (as above) to remove the enzyme. The eluate and washings from this column (40 ml) were diluted to give a conductivity of less than 500 μS, and the pH of the solution was adjusted to 7 with aqueous ammonia (ca. 1 M). The resulting solution was subjected to chromatography on a column (20 cm × 1.5 cm^2) of DEAE-cellulose (DE 52) equilibrated with triethanolamine-HCl buffer, pH 7.35 (5 mM), at 4 °C. The column was washed with this buffer and then eluted with a linear concentration gradient (80 ml + 80 ml) of 5 mM to 300 mM triethanolamine-HCl buffer, pH 7.35. Fractions (1.2 ml) were collected and acidified immediately with 1 M HCl. Samples (50 μl) were taken for scintillation counting, and portions (200 μl) for assay of dihydroxyacetone phosphate and of 3-phosphoglycerate (as described in Herlihy et al. (1976)). In some experiments, the mixture of triose phosphates was not separated, but taken directly for scintillation counting and assay of dihydroxyacetone phosphate (when an approximate value for Φ_S is obtained).

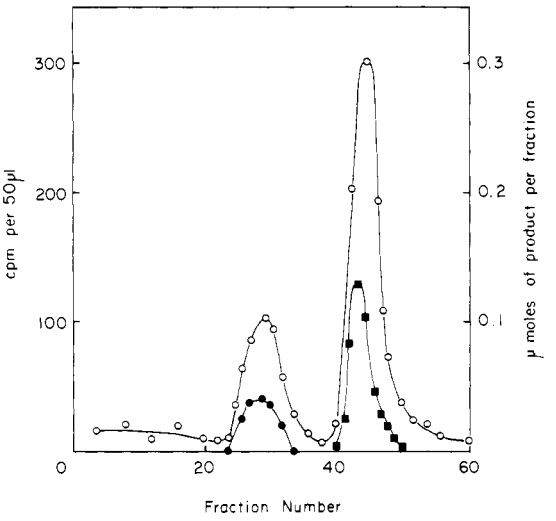


FIGURE 1: Separation of glycerol phosphate and glyceraldehyde 3-phosphate on Dowex 1 (HSO_3^-). The column was eluted with an HCl gradient (pH 3.8 to 0). (●) Glycerol phosphate; (■) glyceraldehyde 3-phosphate; (○) radioactivity. For experimental details, see the text.

Determination of Specific Radioactivity. D-Glyceraldehyde 3-phosphate was assayed by conversion to dihydroxyacetone phosphate and reduction to glycerol phosphate, using isomerase and glycerophosphate dehydrogenase. The assay solution contained 200 mM triethanolamine-HCl buffer, pH 7.6, NADH (0.28 mM), glyceraldehyde 3-phosphate (200 μl sample), in a final volume of 2.515 ml. Glycerophosphate dehydrogenase (10 μl of a solution of 2 mg/ml) was added and the reaction initiated with triosephosphate isomerase (5 μl of a solution of 1 mg/ml). The absorbance change at 340 nm was monitored, an extinction coefficient of 6220 M^{-1} cm at this wavelength being assumed (Horecker and Kornberg, 1948). To check if any bisulfite in the fractions of glyceraldehyde phosphate could upset the assay (by reaction with NAD^+), NAD^+ was added to an assay mixture before initiation of the reaction by the addition of the enzymes. No increase in absorbance at 340 nm was observed.

sn-Glycerol 3-phosphate was assayed by oxidation to

[1] Abbreviations used: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; EDTA, ethylenediaminetetraacetic acid.

TABLE I: The Dependence of the Incorporation of Tritium into the C-2 Position of Remaining Glyceraldehyde 3-Phosphate on the Extent of the Isomerase-Catalyzed Reaction.

Fractional Extent of Reaction[a] $(1-r)$	Spec. Radioact. of Solvent (cpm/μatom) (x)	Spec. Radioact. of Remaining Glyceraldehyde 3-Phosphate[b] (cpm/μmol) (p)	Isotopic Content of Remaining Glyceraldehyde 3-Phosphate[c] (p/x)	B_6'[d]
0.30 ± 0.02	475 600	73 640	0.155 ± 0.01	1.18
0.50 ± 0.02	420 800	94 250	0.22 ± 0.02	1.14
0.60 ± 0.02	508 600	157 150	0.31 ± 0.03	1.14
0.62 ± 0.02	369 000	129 000	0.35 ± 0.03	1.19
0.70 ± 0.02	407 700	142 680	0.35 ± 0.04	1.12
0.70 ± 0.02	233 600	105 000	0.45 ± 0.05	1.16
0.81 ± 0.03	298 000	133 000	0.44 ± 0.05	1.12
0.90 ± 0.02	368 000	160 070	0.435 ± 0.05	1.08

[a] Errors quoted are estimates. [b] These values represent the mean values for the specific radioactivities of the fractions containing glyceraldehyde 3-phosphate. [c] As a fraction of that of the solvent, i.e. (specific radioactivity of remaining glyceraldehyde 3-phosphate)/(specific radioactivity of solvent). The errors quoted are estimates. [d] From eq 3, for $u = 2.5$. Mean value of B_6', 1.14 ± 0.01.

TABLE II: The Incorporation of Tritium into Product *sn*-Glycerol 3-Phosphate during the Isomerase-Catalyzed Reaction of Glyceraldehyde 3-Phosphate.

Fractional Extent of Reaction[a] $(1-r)$	Spec. Radioact. of Solvent (cpm/μatom) (x)	Spec. Radioact. of Glycerol Phosphate[b] (cpm/μmol) (s)	Isotopic Content of Product[c] (s/x)	Corr. for Incomplete Exchange[d] (c)	B_8'[e]
0.62 ± 0.02	369 000	42 440	0.12 ± 0.01	0.005	0.125
0.70 ± 0.02	233 600	25 700	0.11 ± 0.01	0.004	0.114
0.81 ± 0.02	298 000	44 700	0.15 ± 0.01	0.003	0.153

[a] Errors quoted are estimates. [b] These values represent the mean values for the specific radioactivities of the fractions containing glycerol phosphate. [c] This represents the tritium content of product glycerol phosphate compared with that of the solvent and is (specific radioactivity of product)/(specific radioactivity of solvent). The errors quoted are estimates. [d] See text. [e] Mean value of B_8', 0.13$_1$ ± 0.01.

dihydroxyacetone phosphate with glycerophosphate dehydrogenase and NAD^+ in the presence of hydrazine. The presence of hydrazine forces the equilibrium toward the hydrazone of dihydroxyacetone phosphate. Further, the reaction is done at high pH which favors the reduction of NAD^+. The assay solution contained 1 M glycine, 0.4 M hydrazine, and EDTA (4 mM), the pH being adjusted to 9.4 with NaOH, NAD^+ (3.2 mM), and glycerol phosphate (200 μl sample), in a final volume of 2.505 ml. The reaction was initiated with glycerophosphate dehydrogenase (5 μl of a solution of 10 mg/ml) and was followed spectrophotometrically at 340 nm. Control experiments showed that added bisulfite (which may elute from the chromatography column along with glycerol phosphate) does not significantly affect these assays unless the glycerol phosphate concentration is very low (<0.05 mM).

Radioactivity determinations were normally made on duplicate samples (50 μl) from column fractions.

Results

It is essential for the validity of the experiments reported here that the rate of production of glycerol phosphate from glyceraldehyde phosphate (via dihydroxyacetone phosphate) is governed only by the isomerase-catalyzed reaction. The conditions used were at least as stringent as those determined earlier (Plaut and Knowles, 1972). The second requirement is that the isomerase be inactivated at all stages subsequent to the quenching, and the methods used to ensure this have been described in the previous paper (Maister et al., 1976).

The separation of remaining substrate (glyceraldehyde phosphate) and of product (glycerol phosphate) was achieved by exploiting the ability of glyceraldehyde phosphate to form a bisulfite adduct whose charge causes it to be retained more strongly on anion-exchange columns (see Huff, 1959). Since bisulfite may interfere with the assays, a bisulfite gradient was not used, and it was found adequate simply to have the column in the bisulfite form, eluting the components with a pH gradient. A typical separation is shown in Figure 1.

The values of the specific radioactivities of the glycerol phosphate and of the remaining glyceraldehyde phosphate as a function of the extent of the reaction are shown in Tables I and II, respectively. Because of the uncertainty in the assay of low glycerol phosphate levels, the product specific activity was only studied for reactions in which at least 50% of the substrate had been consumed.

The values of the specific radioactivities of the two triose phosphates after equilibration with tritiated water in the presence of the isomerase are given in Table III. These values provide the equilibrium fractionation factors for dihydroxyacetone phosphate (Φ_S) and glyceraldehyde 3-phosphate (Φ_P).

TABLE III: The Specific Radioactivities of Dihydroxyacetone Phosphate and of D-Glyceraldehyde 3-Phosphate after Equilibration by the Isomerase in Tritiated Water.

	Spec. Radioact. of solvent (cpm/μatom)	Spec. Radioact. of Triose Phosphate (cpm/μmol)	Equilibrium Fractionation Factor
			Φ_S
Dihydroxyacetone phosphate	51 400	55 000	1.07 ± 0.04
	156 000	162 500	1.04 ± 0.04
	345 500	342 000	0.99 ± 0.04
			Φ_P
D-Glyceraldehyde 3-phosphate	345 500	345 000	1.00 ± 0.20

TABLE IV: Values of B_6' and B_7' for Various Values of u.

u	B_6' [a]	B_7' [b]	B_7' [d]
1.0	1.41 ± 0.03	0.41 ± 0.03	0.41
2.0	1.18 ± 0.02	0.36 ± 0.03	0.36
3.0	1.12 ± 0.01	0.35 ± 0.03	0.35
4.0	1.09 ± 0.01	0.345 ± 0.03	0.347
∞	1.00	0.33[c]	0.33

[a] Calculated from eq 3, the data of Table I, and u. The errors are standard errors. [b] Calculated from eq 2, B_6', and u. The errors are standard errors. [c] Calculated for $B_6' = 1.0$ from Table III of Albery and Knowles (1976a). [d] Calculated from eq 4 of this paper.

Discussion

Isotopic Content of Remaining Substrate. As has been argued in the previous paper (Maister et al., 1976), the fact that solvent tritium can "enter" into the reaction catalyzed by triosephosphate isomerase only by rapid proton exchange of a reaction intermediate allows us to study the partitioning of this intermediate between starting material and product. Provided that the reaction runs irreversibly and free product (in this case, dihydroxyacetone phosphate) is removed as quickly as it is formed, then the dependence of the specific radioactivity of the remaining substrate on the extent of the reaction measures the partition ratio of the exchanging intermediate. Plots of the form illustrated in Figure 3 of Albery and Knowles (1976a) are expected. When the results from the exchange vs. conversion experiment (Table II) are displayed graphically (Figure 2), it is seen that tritium is indeed incorporated into remaining substrate, which indicates that there is some equilibration of substrate with the solvent [via the enzyme-bound enediol intermediate (see Scheme II of Maister et al. (1976)]. Qualitatively, from the initial gradient of the line in Figure 2, we can say that conversion to product is about three times faster than reversion of the exchanged intermediate to substrate.

The curvature of Figure 2 is much less pronounced than that of the corresponding exchange vs. conversion plot for the reaction of dihydroxyacetone phosphate (Figure 1a of Maister et al., 1976). Whereas this latter curve had the shape expected for "accumulation" (i.e., where isotope from the solvent concentrates on a site in remaining substrate), the shape of the curve in Figure 2 of the present paper corresponds to an "intermediate" case. These terms and the possible shapes of exchange vs. conversion plots have been discussed by Albery and Knowles (1976a) and typical curves are drawn in Figure 3 of that paper. The quantitative expression that describes the present exchange vs. conversion experiment (TP'**p**) is:

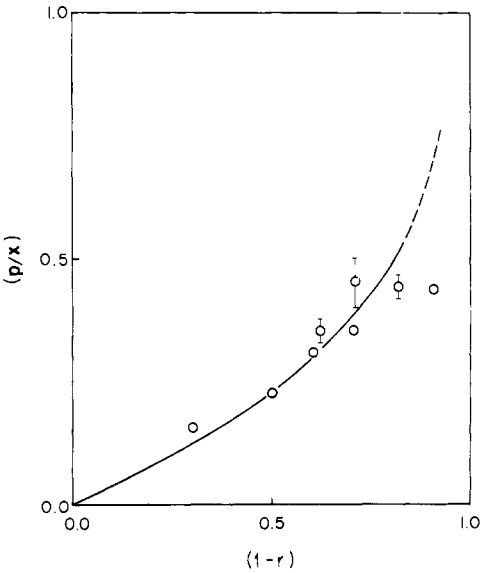


FIGURE 2: Incorporation of ^3H from tritiated water into remaining substrate glyceraldehyde 3-phosphate as a function of the extent of the reaction. The isotopic content (**p**/**x**) of the remaining substrate is plotted as a function of conversion ($1 - r$). The solid line is derived from eq 1 for $B_6' = 1.14$ and $B_7' = 0.35$.

$$\mathbf{p}/\mathbf{x} = B_7'(1 - r^{B_6'-1})/(B_6' - 1) \quad (1)$$

where **p** is the specific radioactivity of the substrate, **x** is the specific radioactivity of the solvent, r is the fraction of remaining reactant, and B_n' are functions of rate constants and fractionation factors defined in Table IV of Albery and Knowles (1976a). The "intermediate" shape of the curve means that $1 < B_6' < 2$.

In Maister et al. (1976), we solved the equation analogous to eq 1 (above), by using a value for A_6' derived from the results of Herlihy et al. (1976). In the present case, however, we have no value for B_6' since we do not have the results of the relevant experiments (TP'**s** and TP'**p**) that are required to find B_6'. These experiments have not been done since the practical difficulties of preparing specifically and stereospecifically labeled D-[2-^3H]glyceraldehyde 3-phosphate of high specific radioactivity would be even greater than those experienced in the preparation of [1(R)-^3H]dihydroxyacetone phosphate. The low extent of tritium transfer observed in the TS'**p** experiment reported by Herlihy et al. (1976) and the difficulties of proving the validity of the values for the extent of tritium transfer in this experiment precluded an attempt on the TP'**s** and TP'**p** experiments as a way of finding B_6'.

The data in Figure 2 are not precise enough to allow us to determine both B_6' and B_7'. However, we can obtain pairs of values of B_6' and B_7' that fit with the results in Figure 2, as follows. If we write:

$$B_7'/(B_6' - 1) = u \quad (2)$$

then eq 1 becomes:

$$B_6' - 1 = \frac{\ln(1 - \mathbf{p}/\mathbf{x}u)}{\ln r} \quad (3)$$

By taking different values of u we can calculate for each value of u, a value of B_6' (fitting the data to eq 3) and thence a value

of B_7' (from eq 2). Table IV gives values of B_6' and B_7' obtained in this way for various values of u. The curve in Figure 2 is drawn for a typical pair of values. The range of u values is fixed first by the fact that the shape of Figure 2 is "intermediate" (Albery and Knowles, 1976a) and secondly by the estimation of u that can be made from the values of A_n' from Herlihy et al. (1976) and Maister et al. (1976). Thus using eq 7.1 of Albery and Knowles (1976a) we find $B_6' \approx 1.14$ and $B_7' \approx 0.3$, which gives (from eq 2) $u \approx 2$. It is satisfactory that, for $u \approx 2$, the values of B_6' and B_7' obtained from the data in Figure 2 and given in Table IV agree well with the estimates of these parameters from the quite different experiments of Herlihy et al. (1976) and Maister et al. (1976). The range of values of B_6' and B_7' in Table IV is the acceptable range for fitting the results of Figure 2. These data may be expressed by eq 4, which simply describes the pairs of values of B_6' and B_7'.

$$B_7' = 0.33\ (\pm\ 0.03) + 0.19(B_6' - 1) \qquad (4)$$

The success of this empirical relationship is shown in the third column of Table IV. Thus while the present results do not permit the determination of both B_6' and B_7', we have obtained eq 4 from Figure 2 and the condition that $1 < B_6' < 2$. The further evaluation of B_6' and B_7' is discussed subsequently (Albery and Knowles, 1976b).

Isotopic Content of Product. With dihydroxyacetone phosphate as the substrate for the triosephosphate isomerase reaction, we have shown (Maister et al., 1976) that, when the reaction is run in tritiated water, the specific radioactivity of the product is close to that of the solvent. The isotopic discrimination against tritium was found to be only 1.3-fold. This showed that the most important product-determining step was not the proton transfer, but the subsequent "substrate-off" step. The difference in the rates of hydrogen and tritium transfer (k_H and k_T in Scheme II of Maister et al., 1976) is nullified by the fact that the enzyme-bound enediol intermediate and the enzyme-bound product are in (or close to) equilibrium with one another. In the present paper we have measured the isotopic discrimination against tritium for the reaction in the other direction, with glyceraldehyde 3-phosphate as substrate and glycerol phosphate as the final product. From Table II we see that the discrimination against tritium is now about ninefold. This is in the range of an acceptable primary kinetic isotope effect and indicates that the product-forming step in the direction glyceraldehyde 3-phosphate → dihydroxyacetone phosphate ⇒ *sn*-glycerol 3-phosphate involves isotope transfer. That is, whereas the difference between k_H and k_T is irrelevant when the reaction (Scheme II of Maister et al., 1976) is run from left to right because k_{off} is slow and A, B, C and D are all in equilibrium with one another, when the reaction is run from right to left we *do* see the difference between k^H and k^T in the formation of product.

The quantitative treatment of the data in Table II uses the equation for the TPs experiment (T, tritium; P, initially *un*labeled glyceraldehyde phosphate as substrate in tritiated solvent; **s**, analyze for isotope in the product S_2) (see Table III of Albery and Knowles, 1976a):

$$\mathbf{s}/\mathbf{x} = B_8' - c \qquad (5)$$

where

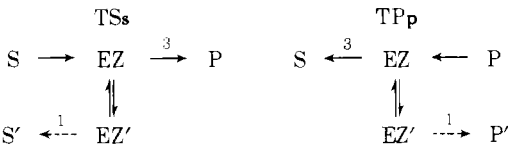
$$c = uB_5'[(r - r^{B_6'})/(1 - r)] \qquad (6)$$

and **s** is the specific radioactivity of the product, **x** is the specific radioactivity of the solvent, and u is defined by eq 2.

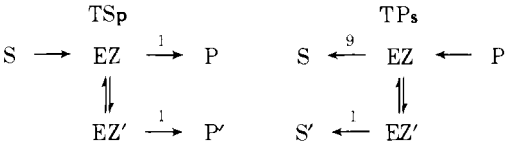
From eq 7.1 of Albery and Knowles (1976a) and the values of A_n' in Herlihy et al. (1976) and Maister et al. (1976), we find that $B_5' \approx 0.02$. Using values of B_6' and u from Table IV we then obtain values for c (from eq 6) as shown in Table II. It is evident that c is small, and that the value of B_8' from eq 5 is insensitive to the choice of u and B_6' in determining c. The derived values of B_8' are listed in Table II.

The Approximate Position of the Energy Barriers. In a subsequent paper (Albery and Knowles, 1976b) we analyze in detail the A_n and B_n parameters we have obtained, but at this stage we can already discern some of the principal features of the catalyzed reaction from the experiments reported in this paper and in Maister et al. (1976).

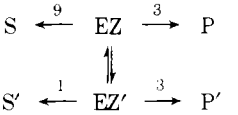
In the exchange vs. conversion experiment with dihydroxyacetone phosphate as substrate (TSs: Maister et al., 1976), the *initial* gradient of the exchange-conversion plot showed that the enediol intermediate is converted to product about three times as fast as it collapses back (picking up ^3H) to labeled substrate. Similarly, when glyceraldehyde phosphate is the substrate (TPp: this paper), the initial gradient of Figure 2 shows that the intermediate is converted to product about three times as fast as it reverts back to labeled substrate. The situation can be represented schematically:



(the above is an abbreviated form of Scheme I of Albery and Knowles, 1976a: S represents dihydroxyacetone phosphate; EZ is the enediol intermediate; P is glyceraldehyde phosphate; primes indicate labeled species). The two experiments in which the *products* are analyzed may be similarly expressed:



The approximate ratio of 1 in the TSp experiment may be deduced from the absence of any substantial discrimination against tritium in the S → P direction (Maister et al., 1976), while the ratio of 9 in the TPs experiment arises from the results shown in Table II. We may now derive a single scheme to describe the fate of protonated and tritiated enediol intermediate (EZ and EZ′):



This scheme contains *three* ratios that explain the *four* independently determined ratios given above and provides gratifying confirmation of the mechanistic and energetic deductions made so far. Furthermore, by using four tritium experiments we have found the 3:1 ratio for the partitioning of EZ in the all-hydrogen system: the enediol (EZ) goes three times as often to dihydroxyacetone phosphate (S) as it does to D-glyceraldehyde 3-phosphate (P).

Acknowledgment

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Energetics of Triosephosphate Isomerase: Deuterium Isotope Effects in the Enzyme-Catalyzed Reaction†

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ABSTRACT: The effect of isotopic substitution of the specifically labilized hydrogen in the substrates of triosephosphate isomerase on the steady-state rates of the enzyme-catalyzed reaction has been examined. The k_{cat} value for the enzyme-catalyzed transformation of [1(*R*)-^{2}H]dihydroxyacetone phosphate is 2.9 times smaller than that for the 1(*R*)-^{1}H compound. Because of the rapid loss of ^{2}H to solvent from the enzyme–enediol complex, this factor represents the full kinetic isotope effect of the proton abstraction step. The values of k_{cat} and of K_m for D-[2-^{2}H]glyceraldehyde 3-phosphate are indistinguishable from those of the 2-^{1}H material. This arises from the rapid loss of ^{2}H from the enzyme–enediol intermediate, which results in ^{1}H rather than ^{2}H transfer in the rate-limiting step. The steady-state kinetic results reported in this paper qualitatively confirm and quantitatively extend the results from the previous papers on the variation of the free energy along the reaction path.

The use of tritium labeling as a mechanistic probe for the reaction catalyzed by triosephosphate isomerase has been described in the previous three papers (Herlihy et al., 1976; Maister et al., 1976; Fletcher et al., 1976). The use of this hydrogen isotope is normally confined to competitive situations, in which isotope effects in product-forming steps become apparent from the discrimination between hydrogen and tritium in the product under scrutiny. This approach is particularly useful for the investigation of reaction steps that may not be rate limiting in the overall reaction. Deuterium can often be used in a similar manner, but in addition, the use of deuterium-labeled substrates provides information about the importance of the particular protonation and deprotonation steps in the actual rate of the overall process (see Figure 5 of Albery and Knowles, 1976a).

We present here the steady-state parameters for the reaction of the two specifically deuterated substrates, [1(*R*)-^{2}H]-dihydroxyacetone phosphate and D-[2-^{2}H]glyceraldehyde 3-phosphate, with triosephosphate isomerase.

Experimental Section

Materials. Enzymes, substrates, cofactors, and other materials were as specified by Herlihy et al. (1976). Additionally, phosphoenolpyruvate (monopotassium salt) and pyruvate kinase (from rabbit muscle, as a crystalline suspension in ammonium sulfate) were obtained from the Sigma Chemical Co. (London, England). ^2H_2O (>99.8% isotopic purity) was obtained from Norsk Hydroelektrisk (through Rivan Chemicals, Southampton, England).

[*1(R)-^{2}H*]*Dihydroxyacetone phosphate* was prepared as follows. Dowex 50 (H^+ form, 100–200 mesh, 4% cross-linked), 3.5 g, was washed thoroughly with ^2H_2O. The precursor of dihydroxyacetone phosphate (dicyclohexylammonium 2,2-dimethoxy-1,3-propanediol phosphate), 25 mg, was dissolved in ^2H_2O (2 ml) and swirled with the washed resin for 30 s, and the resin was then removed by rapid filtration. The resin was washed with small portions of ^2H_2O and the combined filtrates were incubated at 37 °C for 4.5 h. The "pH" of the solution was then adjusted to ca. 7.5 by the addition of anhydrous K_2CO_3. To this solution containing dihydroxyacetone phosphate (48 μmol) in ^2H_2O (3 ml) was added triosephosphate isomerase (5 μl of a solution of 16 mg/ml) and the mixture left at 20 °C for 1.5 h. All operations up to this point were performed in stoppered vessels with guard tubes to prevent the incursion of atmospheric moisture. After the incubation with isomerase, the solution was cooled to 0 °C and the "pH" lowered rapidly to about 4 by the addition of 1 M HCl. The mixture was then applied to a column (15 × 1.5 cm^2) of Dowex 1 (Cl^- form, 200–400 mesh, 8% cross-linked) equilibrated at 4 °C with 0.16 mM HCl, pH 3.8. The column was washed with

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.