Rieder, S. V., and Rose, I. A. (1959), *J. Biol. Chem. 234,* 1007-1010.
Rose, I. A. (1962), *Brookhaven Symp. biol. 15,* 293-309.
Rutter, W. J. (1961), *Enzymes, 2nd Ed. 5,* 341-366.
Simon, H., Medina, R., and Müllhofer, G. (1968), *Z. Naturforsch. 23,* 59-64.
Steitz, T. A., Henderson, R., and Blow, D. M. (1969), *J. Mol. Biol. 46,* 337-338.
Veech, R. L., Raijman, L., Dalziel, K., and Krebs, H. A. (1969), *Biochem. J. 115,* 837-842.

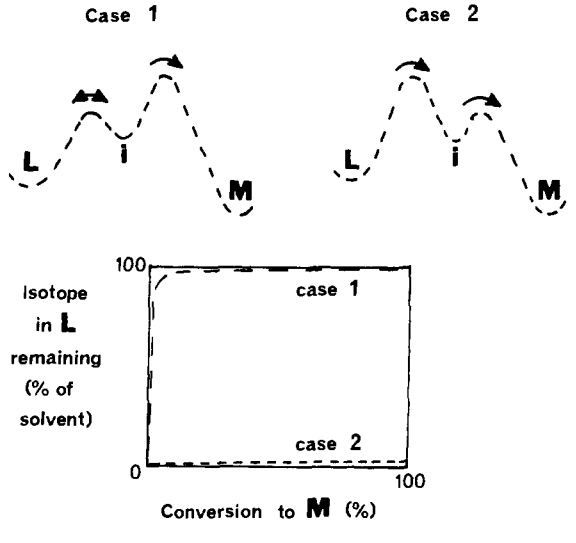
Energetics of Triosephosphate Isomerase: The Appearance of Solvent Tritium in Substrate Dihydroxyacetone Phosphate and in Product†

Selwyn G. Maister, Christopher P. Pett, W. John Albery,‡ and Jeremy R. Knowles*

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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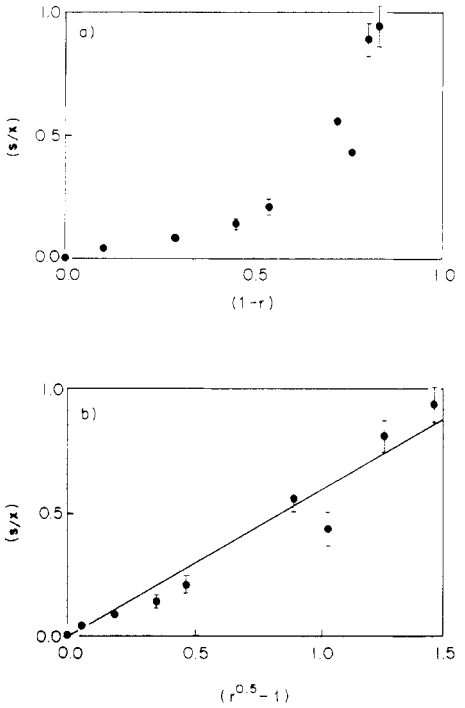


FIGURE 1: (a) Incorporation of ^3H from tritiated water into remaining substrate dihydroxyacetone phosphate as a function of the extent of the reaction. The specific radioactivity of remaining dihydroxyacetone phosphate is s, and of the solvent is x. The fractional extent of the reaction is $1 - r$; the fraction of remaining reactant is r. (b) Plot of the data of Figure 1a according to eq 1. A_6' is 0.50, derived from Herlihy et al. (1976).

Experimental Section

Materials. Enzymes, substrates, cofactors, and other materials were as described by Herlihy et al. (1976).

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

Isomerase-Catalyzed Reactions. Isomerase-catalyzed reactions of dihydroxyacetone phosphate were performed in a 10-mm light-path optical cuvette, in a total volume of 2.601 ml, containing: 100 mM triethanolamine-HCl buffer (pH 7.6), EDTA[1] (20.8 mM), sodium arsenate (3.8 mM), glyceraldehyde-phosphate dehydrogenase (0.77 mg/ml), NAD^+ (2.4 mM), dihydroxyacetone phosphate (0.92 to 1.1 mM), and tritiated water (20 mCi/ml). The reaction was initiated with triosephosphate isomerase (0.19 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 monitored at 340 nm, an extinction coefficient for NADH of 6220 M^{-1} cm being assumed (Horecker and Kornberg, 1948). The reaction was stopped after the desired extent of reaction had been reached, by lowering the pH to below 4 by the addition of 1 M HCl (140 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).

Separation Methods. Early attempts to separate dihydroxyacetone phosphate and 3-phosphoglycerate both from each other and from non-carbon-bound ^3H centered on Dowex 1 as used by Rose and Rieder (1958). The quenched reaction mixture was applied directly to a column (4 cm × 1.7 cm^2) of Dowex 1 (Cl^-) previously equilibrated with 0.16 mM HCl (pH 3.8). The column was washed with up to 70 column volumes of 0.16 mM HCl to remove all labile ^3H, until the radioactivity in the eluate was near to background. A nonlinear pH gradient was then applied (25 + 25 ml; 0.16 mM HCl to 0.1 M HCl), and 1-ml fractions were collected. This procedure did not produce consistent or satisfactory separation of dihydroxyacetone phosphate and 3-phosphoglycerate. Efforts to improve the separation and to reduce the level of background radioactivity were made as follows. A column of Dowex 1 in the acetate form at pH 4.1 was eluted with a linear gradient of sodium acetate (5–500 mM) at the same pH, but it was found that the phosphate esters did not bind to the column. Accordingly, having applied the phosphate esters to a Dowex 1 (Cl^-) column at pH 3.8, elution with a linear gradient of potassium chloride at the same pH was attempted. It was found that the separation of esters was not significantly better than that with the pH gradient used first. Finally it was found that the use of DEAE-cellulose (DE 52) at pH 8.1, eluted with a linear gradient of ammonium bicarbonate, gave consistent and good separation of dihydroxyacetone phosphate and 3-phosphoglycerate. The following procedure was adopted. After quenching the reaction mixture at pH 3.8, most of the ^3H_2O was removed by freeze-drying. To ensure complete removal of isomerase, the freeze-dried residue was dissolved in a small volume (0.5 ml) of 0.16 mM HCl and passed through a small column (2 cm × 1.7 cm^2) of Dowex 50 (H^+) at pH 3.8. The pH of the eluate (25 ml) was raised to pH 7.6 with ammonium hydroxide and the solution diluted with deionized water to 40 ml. This solution was applied to a column of DEAE-cellulose (6 cm × 1.7 cm^2) equilibrated with 5 mM ammonium bicarbonate, pH 8.1, at 4 °C. The column was washed with 5 mM ammonium bicarbonate to remove any traces of tritiated water. A linear gradient (80 ml) of ammonium bicarbonate (5–300 mM) was then applied to the column, and fractions of ca. 1 ml were collected. The pH of each fraction was lowered by the addition of 3 drops of 1 M HCl immediately after collection. Under these conditions, good yields of completely separated dihydroxyacetone phosphate and 3-phosphoglycerate were obtained. [Higher yields of dihydroxyacetone phosphate with no loss in column resolution can be achieved using a lower pH of 7.1 for the column (see Herlihy et al. (1976), Figure 1).]

Determination of Specific Radioactivity. Dihydroxyacetone phosphate was assayed by reduction to glycerol phosphate with α-glycerophosphate dehydrogenase. The solution contained 200 mM triethanolamine-HCl buffer (pH 7.6), NADH (0.28 to 0.028 mM), and dihydroxyacetone phosphate (50 μl sample), in a final volume of 1.251 ml. The reaction was initiated by the addition of the dehydrogenase (1 μl of a solution of 2 mg/ml) and the absorbance change at 340 nm was monitored.

3-Phosphoglycerate was assayed by phosphorylation by phosphoglycerate kinase to 1,3-diphosphoglycerate followed by reductive dephosphorylation with glyceraldehyde-phosphate dehydrogenase to glyceraldehyde 3-phosphate. The presence of hydrazine forces the equilibria toward the hydrazone of glyceraldehyde phosphate. The solution contained 200 mM triethanolamine-HCl buffer (pH 7.6), EDTA (5 mM), $MgSO_4$ (10 mM), ATP (6 mM), NADH (0.17 mM), hydrazine hydrochloride (1.6 mM), glyceraldehyde-3-phosphate dehydrogenase (100 μg/ml), and 3-phosphoglycerate (200 μl sample) in a final volume of 2.6 ml. The reaction was initiated

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

by the addition of phosphoglycerate kinase (5 μl of a solution of 2 mg/ml) and the absorbance change at 340 nm was monitored. Duplicate assays of both dihydroxyacetone phosphate and 3-phosphoglycerate were performed.

Radioactivity determinations were normally made directly on duplicate samples (100 μl) of the column eluate. This method is satisfactory, provided that the background level of radioactivity eluting from the column is low, and provided that it can be assumed that the ^3H in the phosphate ester is attached only to the expected carbon atom. Each of these points was checked as follows. For dihydroxyacetone phosphate, appropriate fractions from the column were pooled together in pairs and freeze-dried from a 5-ml flask. The apparatus consisted of a short bridge to which two 5-ml flasks could be attached, with a side-arm and tap, leading to the vacuum pump. Both flasks were cooled in liquid N_2 and the system was evacuated. After isolation from the pump (using the tap on the side-arm), the flask containing the sample was removed from the liquid N_2 and the water allowed to distill into the other flask. Deionized water was then added to the sample, and the distillation was repeated until no further ^3H_2O distilled over. The sample flask could now be assayed for nonvolatile radioactivity, and for dihydroxyacetone phosphate. This technique eliminates the problem of high ^3H_2O backgrounds. In order to check that the ^3H in the dihydroxyacetone phosphate is actually bound to the 1(*R*) position, the involatile residue was dissolved in 200 mM triethanolamine buffer (0.5 ml), pH 7.6, and triosephosphate isomerase (5 μl of a solution of 17 mg/ml) was added. After incubation at 20 °C for 1 h, 1 M HCl (40 μl) was added, and the ^3H_2O was distilled over by the technique described above. Deionized water was then added to the sample and the distillation repeated. In this way, only the ^3H label in the dihydroxyacetone phosphate that can be labilized by isomerase is washed out into the solution as ^3H_2O. The most rigorous precautions against adventitious ^3H_2O (e.g., from the vacuum line or pump) must be taken in these experiments. It was shown that the radioactivity in the dihydroxyacetone phosphate was indeed attached to the ester at the 1(*R*) position (see Table I). For 3-phosphoglycerate, the location of ^3H on C-2 only could be demonstrated by a similar method involving the "washing out" of ^3H at this position into the solvent by incubation of the 3-phospho[2-^3H]glycerate with phosphoglyceromutase, enolase, and 2,3-diphosphoglycerate. This is described fully by Herlihy et al. (1976).

TABLE I: Confirmation That Radioactivity in Remaining Dihydroxyacetone Phosphate Is in the 1(*R*) Position.[a]

(1) Total cpm in pooled, freeze-dried fractions containing dihydroxyacetone phosphate	5778 cpm
(2) Total amount of dihydroxyacetone phosphate in (1)	0.586 μmol
(3) App spec radioactivity of dihydroxyacetone phosphate [(1)/(2)]	9850 cpm/μmol
(4) Total cpm taken for incubation with isomerase	3620 cpm
(5) Total cpm in water distilled after isomerase incubation	3900 cpm
(6) Total cpm in residue after isomerase incubation	280 cpm
(7) % radioactivity in the 1(*R*) position of dihydroxyacetone phosphate [100 × (5)/(4)]	108 %

[a] For details, see Experimental Section. The results in this table relate to an experiment that proceeded to 29% conversion.

Results

One of the prerequisites for the validity of this work is that the reaction of dihydroxyacetone phosphate via glyceraldehyde phosphate to 3-phosphoglycerate is cleanly rate limited by the isomerase-catalyzed step. If free glyceraldehyde phosphate is produced faster than it can be consumed by the dehydrogenase, the isomerase will have the opportunity to equilibrate the triose phosphates with the tritiated water, which will vitiate any deductions made from the appearance of ^3H in substrate and product. In these experiments, the activity ratio of dehydrogenase:isomerase was always greater than 10, and the reaction rate was limited by isomerase (Plaut and Knowles, 1972). Addition of larger excesses of dehydrogenase had no effect on the reaction rates.

A second requirement is that isomerase be inactivated or removed after the reaction has been stopped at the appropriate stage. Quenching of the reaction by lowering the temperature to 0 °C and the pH to below 4 is known to inactivate the enzyme (Plaut and Knowles, 1972) and, to guard against any possible reactivation at neutral pH values, all operations were carried out below pH 4 until the isomerase had been removed.

The separation of dihydroxyacetone phosphate and 3-phosphoglycerate on columns of Dowex 1 with a pH gradient from pH 4 to 1, although it has been used previously, was not adequately reproducible in our hands. So, despite the two advantages (a) that, after quenching, the isomerase never reaches a pH where it could be active, and (b) that dihydroxyacetone phosphate is most stable at low pH, the separation method finally adopted used a DEAE-cellulose column at pH 8, and elution with a salt gradient. At this pH, the unit difference in charge between the two esters could be exploited. Enzymes were removed completely after quenching by passage down a small column of Dowex 50, and the subsequent separation produced the phosphate esters in good yield, with low background radioactivity in the column eluate. A typical separation is shown in Figure 1 of Herlihy et al. (1976).

The values of the specific radioactivity for both 3-phosphoglycerate and for dihydroxyacetone phosphate, as a function of the extent of the reaction, are shown in Tables II and III, respectively. It is evident that the specific activity of the product 3-phosphoglycerate is independent of the extent of reaction, being approximately constant at about 0.77 that of the solvent. In contrast, the specific radioactivity of the substrate dihydroxyacetone phosphate varies with the extent of reaction (Table III). This dependence is presented in Figure 1a. (It may be noted that the form of Figure 1a differs from that of preliminary experiments performed at room temperature reported by Knowles et al. (1971). When, as here, the energy barriers for successive steps in a multistep reaction are so close, it is clearly important that all experiments be performed under the same conditions, particularly of temperature and pH.)

Discussion

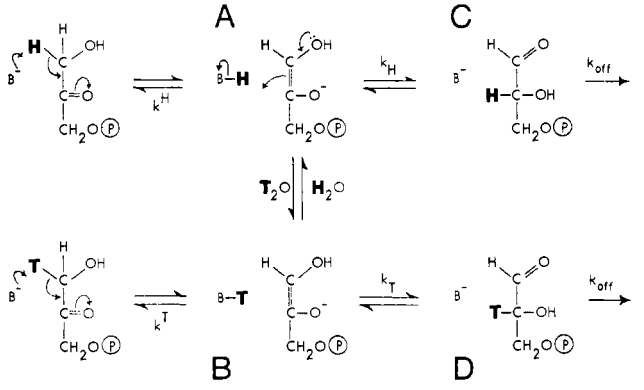
On the basis of the isotope exchange and incorporation work done by Rieder and Rose (1956, 1959) and by Bloom and Topper (1956, 1958), Rose (1962) proposed a chemically attractive pathway for the ketose-aldose isomerases, involving proton abstraction from either substrate to give an enzyme-bound enediol intermediate. The stereochemistry of the labilized hydrogen from the prochiral center of the ketose and the stereochemistry of the chiral center (C-2) of the aldose, coupled with the existence (for phosphoglucose isomerase, at least) of a significant amount of direct transfer of hydrogen from C-1 to C-2 (Rose, 1962), led to the suggestion that the enediol has the cis configuration. These proposals are summarized in Scheme II.

TABLE II: The Incorporation of Tritium into Product 3-Phosphoglycerate during the Isomerase-Catalyzed Reaction of Dihydroxyacetone Phosphate.[a]

Fractional Extent of Reaction[b] $(1 - r)$	Spec Radioact. of Solvent[c] (cpm/μatom)	Spec Radioact. of 3-Phosphoglycerate[d] (cpm/μmol)	Isotopic Content of Product[e] (**p**/**x**)	Corr for Incomplete Exchange[f] (c)	A_8'[g]
0.20 ± 0.01	120 100	91 350	0.76 ± 0.04	0.014	0.77
0.31 ± 0.01	126 550	97 090	0.77 ± 0.05	0.013	0.78
0.40 ± 0.02	76 250	55 750	0.73 ± 0.04	0.013	0.74
0.84 ± 0.02	165 000	132 000	0.80 ± 0.04	0.008	0.81
					Mean: 0.78 ± 0.01

[a] For details, see Experimental Section. [b] The extent of reaction was estimated by measuring the absorbance due to NADH at the moment of quenching, knowing the initial concentration of substrate dihydroxyacetone phosphate. The errors quoted are estimates. [c] Duplicate samples (10 μl) of the reaction mixture were taken for scintillation counting before the addition of isomerase initiates the reaction. [d] These values represent the mean values for the specific radioactivities of the fractions from the column containing 3-phosphoglycerate. [e] Isotopic content of the product compares the specific radioactivity of the product 3-phospho[2-^{3}H]glycerate (**p**) with the specific radioactivity of the solvent (**x**). The errors quoted are estimates. [f] Calculated from eq 3 with values of A_6' and A_5' from Herlihy et al. (1976) and A_7' from this paper. [g] Calculated from eq 2. The error is the standard error of the mean.

SCHEME II: The Pathway of the Triosephosphate Isomerase Catalyzed Reaction of Dihydroxyacetone Phosphate.



Isotopic Content of Product. Consider first the reaction of specifically tritiated [1(R)-^{3}H]dihydroxyacetone phosphate. When this is a substrate for isomerase under irreversible conditions (i.e., in the presence of excess glyceraldehyde-phosphate dehydrogenase and cofactors), essentially all the ^{3}H is lost to the medium (Rose, 1962; Herlihy et al., 1976); that is, despite the very rapid conversion of dihydroxyacetone phosphate to glyceraldehyde phosphate [the k_{cat} for this reaction is 430 s^{-1} (Putman et al., 1972)], the conjugate acid of the putative enzyme base exchanges the tritium label almost completely with solvent. In Scheme II, this shows that A and B are in equilibrium with one another (i.e., the enediol intermediate is in isotopic equilibrium with the solvent). Now, k_H (A → C) must be much larger than k_T (B → D) since this step involves the transfer of a proton (or tritium ion) from the enzyme to the enediol and the process must show a primary kinetic isotope effect. We might expect, therefore, that, although A and B are in isotopic equilibrium with solvent, there should be discrimination against tritium in the product, leading to a much lower specific radioactivity in the product, because $k_H > k_T$. (Putting this another way, B/D should be larger than A/C by a factor of say 6–20, equal to the primary kinetic isotope effect.)

This argument is not supported by the results of Table II. The final product 3-phosphoglycerate (and, therefore, the immediate product of isomerase:glyceraldehyde phosphate) has a specific radioactivity 0.77 that of the solvent; that is, the discrimination against tritium is only 1.3-fold. A factor of 1.3 is very much lower than could reasonably arise from a primary kinetic isotope effect for a transition state in which H is actually being transferred. To accommodate this result, the enzyme-bound forms of glyceraldehyde phosphate (C and D) must be in equilibrium with the exchanging enediol intermediate (A and B). This in turn demands that some step after the formation of the enzyme–product complex is the most important rate-limiting step. Only in this way can the lack of isotopic discrimination in the product be accounted for. The slow step could either be the loss of glyceraldehyde phosphate from the enzyme, or some slow conformational change preceding this loss. Since the value of k_{cat}/K_m for the back reaction (with glyceraldehyde phosphate as substrate) is 3×10^8

TABLE III: The Dependence of the Incorporation of Tritium into the 1(R) Position of Remaining Dihydroxyacetone Phosphate, on the Extent of the Isomerase-Catalyzed Reaction.[a]

Fractional Extent of Reaction[b] $(1 - r)$	Spec Radioact. of Solvent[c] (cpm/μatom)	Spec Radioact. of Remaining Dihydroxy-acetone Phosphate[d] (cpm/μmol)	Tritium Content of Remaining Dihydroxyacetone Phosphate[e] (**s**/**x**)
0.100 ± 0.003	91 500	3 610	0.039 ± 0.004
0.29 ± 0.01	124 000	9 985	0.081 ± 0.006
0.46 ± 0.02	101 750	13 815	0.14 ± 0.02
0.54 ± 0.02	116 375	22 070	0.21 ± 0.03
0.73 ± 0.02	105 550	58 150	0.55 ± 0.05
0.76 ± 0.02	111 750	47 250	0.42 ± 0.06
0.805 ± 0.02	128 350	111 300	0.885 ± 0.065
0.835 ± 0.02	129 500	121 000	0.94 ± 0.08

[a] For details, see Experimental Section. [b] The extent of the reaction was estimated by measuring the absorbance due to NADH at the moment of quenching, knowing the initial concentration of dihydroxyacetone phosphate. The errors quoted are estimates. [c] Duplicate samples (10 μl) of the reaction mixture were taken for scintillation counting before the addition of isomerase initiates the reaction. [d] These values represent the mean values for the specific radioactivities of the fractions from the column, containing dihydroxyacetone phosphate. Checks were performed to establish that the radioactivity was associated with dihydroxyacetone phosphate, in the 1(R) position (for details, see text and Table I). [e] The specific radioactivity of dihydroxyacetone phosphate is **s**, and of solvent is **x**. The errors quoted are estimates.

M^{-1} s^{-1} (Putman et al., 1972) which is at the diffusion-controlled limit for such a reaction, it is improbable that any slow conformational change is involved in the binding of glyceraldehyde phosphate to the enzyme, and the slow step is, therefore, just the loss of glyceraldehyde phosphate from the enzyme.

Isotopic Content of Remaining Substrate. It is apparent from Figure 1a that the specific radioactivity of the remaining substrate dihydroxyacetone phosphate increases during the course of the reaction. The fact that tritium is incorporated into substrate at all shows that the energetic situation resembles case 1 of Scheme I, in which there is some preequilibration of substrate with the solvent (via the enzyme-bound enediol intermediate). This is, of course, quite consistent with the deduction made above that the final step in the reaction, the loss of product, is slow. However, Figure 1a contains more information than either of the limiting cases of Scheme I since we can actually see the dependence of isotopic exchange on the extent of reaction and can, therefore, derive the partitioning ratio of the intermediate. From the initial gradient of the curve, it is apparent that the enediol intermediate is converted into product about three times as fast as it collapses back (picking up ^3H) to starting dihydroxyacetone phosphate. Two other features of Figure 1a deserve comment. First, the curve is concave upward, and, secondly, the specific radioactivity of substrate remaining near the very end of the reaction will actually be *higher* than the specific radioactivity of the solvent. These two facts are understandable on the following basis. At the start of the reaction protonated dihydroxyacetone phosphate suffers proton abstraction to give the enediol intermediate (Scheme II). This intermediate now exchanges rapidly with solvent (A $\rightleftharpoons$ B) and equilibrates with the two forms of enzyme-bound product (C and D). These species (which can be considered as one species since all are in equilibrium with one another) can now partition in three ways: to free product (represented by "k_{off}" in Scheme II), or back to protonated (k^H) or tritiated (k^T) substrate. Since k^H is greater than k^T by the primary isotope effect, tritium will appear rather slowly in the substrate, at first. After the reaction has proceeded for some time, there will be a competition between protonated and tritiated substrate which the former will win, because of the isotope effect in the first abstraction to the enediol. As the protonated substrate is preferentially consumed, tritiated substrate will continue to accumulate. Tritiated substrate is, after all, being continuingly supplied by the partitioning of material in the C $\rightleftharpoons$ A $\rightleftharpoons$ B $\rightleftharpoons$ D pool, and simultaneously being discriminated against, in reaching this pool. So the curvature of Figure 1a is concave upward, and it is not surprising that at the last stages of reaction, what little substrate remains has a very high specific radioactivity. At this point in the reaction, the major effect is that of discrimination in favor of protonated substrate, which causes the specific activity of what remains to rise further.

Quantitative Treatment of the Results. The above discussion has merely presented a qualitative physical picture of the phenomena that have been observed. Quantitative information about the reaction can be obtained, however, by the application of the theoretical treatment given in the first paper of this series (Albery and Knowles, 1976a).

According to the nomenclature used earlier (Albery and Knowles, 1976a), the analysis of the tritium content of the reactant is a TSs experiment (T, tritium; S, initially *un*labeled dihydroxyacetone phosphate as substrate in tritiated solvent; **s**, analyze for isotope in the remaining substrate). From Table III of Albery and Knowles (1976a), the equation for this experiment is:

$$\mathbf{s}/\mathbf{x} = A_7'(1 - r^{A_6'-1})/(A_6' - 1) \quad (1)$$

where **s** is the specific radioactivity of the reactant, **x** is the specific radioactivity of the solvent, r is the fraction of remaining reactant, and A_n' are functions of rate constants and fractionation factors defined in Table IV of Albery and Knowles (1976a).

From Herlihy et al. (1976) we know that A_6' is 0.50 ± 0.03, and this value can be used to plot the results in Table III and Figure 1a of this paper according to eq 1. This plot is shown in Figure 1b, which is a reasonable straight line passing through the origin. The fit provides further confirmation of the value of A_6', and yields a value for A_7' of 0.27 ± 0.025. The error quoted is an estimate of the standard error allowing for the uncertainty in the value of A_6'.

The second experiment reported in this paper is a TS**p** experiment (T, tritium; S, *un*labeled dihydroxyacetone phosphate as reactant in tritiated solvent; **p**, analyze for isotope in the product).

From Table III of Albery and Knowles (1976a), for a TS**p** experiment:

$$\mathbf{p}/\mathbf{x} = A_8' - c \quad (2)$$

where

$$c = [A_5'A_7'/(A_6' - 1)][(r - r^{A_6'})/(1 - r)] \quad (3)$$

and **p** is the specific radioactivity of the product. c is a small term describing the effect of incomplete isotopic equilibration of the exchanging reaction intermediate with the solvent. With the knowledge of A_5' and A_6' from Herlihy et al. (1976), and of A_7' from eq 1 (above), c can be calculated (see Table II). As can be seen from Table II, the values of c are small, and **p**/**x** does not vary significantly with the extent of the reaction. The values of A_8' are obtained from eq 2, and result in a mean value of 0.78 ± 0.01 (Table II).

Further analysis of the parameters A_7' and A_8' derived from the experiments described in this paper is presented in Albery and Knowles (1976b).

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References

Albery, W. J., and Knowles, J. R. (1976a), *Biochemistry 15*, first of eight papers in a series in this issue.

Albery, W. J., and Knowles, J. R. (1976b), *Biochemistry 15*, seventh of eight papers in a series in this issue.

Bloom, B., and Topper, Y. J. (1956), *Science 124*, 982-983.

Bloom, B., and Topper, Y. J. (1958), *Nature (London) 181*, 1128-1129.

Herlihy, J. M., Maister, S. G., Albery, W. J., and Knowles, J. R. (1976), *Biochemistry 15*, second of eight papers in a series in this issue.

Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem. 175*, 385-390.

Knowles, J. R., Leadlay, P. F., and Maister, S. G. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36*, 157-164.

Plaut, B., and Knowles, J. R. (1972), *Biochem. J. 129*, 311-320.

Putman, S. J., Coulson, A. F. W., Farley, I. R. T., Riddleston, B., and Knowles, J. R. (1972), *Biochem. J. 129*, 301–310.
Rieder, S.V., and Rose, I. A. (1956), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 15*, 337.
Rieder, S. V., and Rose, I. A. (1959), *J. Biol. Chem. 234*, 1007–1010.
Rose, I. A. (1962), *Brookhaven Symp. Biol. 15*, 293–309.
Rose, I. A., and Rieder, S. V. (1958), *J. Biol. Chem. 231*, 315–329.

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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