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The Uncatalyzed Rates of Enolization of Dihydroxyacetone Phosphate and of Glyceraldehyde 3-Phosphate in Neutral Aqueous Solution. The Quantitative Assessment of the Effectiveness of an Enzyme Catalyst†

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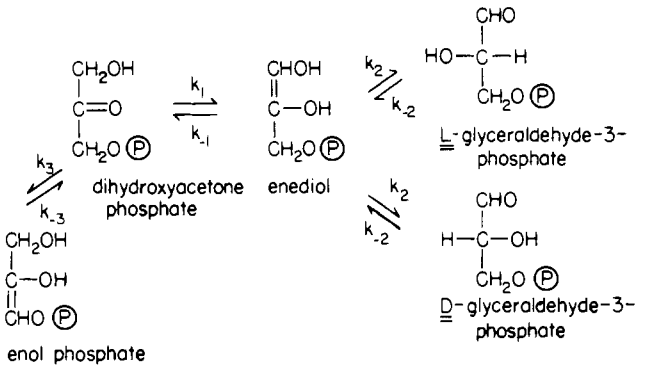
ABSTRACT: By a combination of methods involving enzyme-catalyzed reactions and classical iodination techniques it has been possible to obtain all the relevant rate constants for the uncatalyzed interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate via their common enediol intermediate. These rate constants are compared with those for the individual steps of the triosephosphate isomerase catalyzed reaction, and a quantitative picture of the effectiveness of the enzyme as a catalyst has been delineated. It is apparent that the enzyme increases the enolization rate of dihydroxyacetone phosphate by a factor of more than 10^9 over that of the uncatalyzed reaction.

The kinetics of the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate catalyzed by the glycolytic enzyme triosephosphate isomerase (EC 5.3.1.1.) are well understood, and the rate constants for all the kinetically significant steps of the catalyzed reaction have been determined (W. J. Albery and J. R. Knowles, unpublished work). From these data, it is evident that the isomerase has developed to the point where further evolutionary refinement would have no effect, since the interconversion is diffusion controlled.

In order to compare the catalyzed rates of this simple one-substrate–one-product reaction with the rates of the uncatalyzed process, we need to know the rates of enolization of the two reactants, and the equilibrium percentage of their common enediol. The two reports in the literature of these rates (Reynolds et al., 1971; Grazi et al., 1973) are conflicting, and it seems not to have been appreciated that the enol from glyceraldehyde 3-phosphate is the same as one of the two possible enols from dihydroxyacetone phosphate (Reynolds et al., 1971). Since there is good evidence that the enzyme-catalyzed reaction also involves this common enediol as an intermediate (Rieder and Rose, 1959), it is of some interest to find how the free energy profile (which is simply a convenient way of illustrating and summarizing the rate and equilibrium constants) for the uncatalyzed reaction compares with that for the maximally efficient interconversion catalyzed by triosephosphate isomerase. We report here the results obtained for the rates of the enolization reactions, determined both by the classical method involving iodination of the enediol, and also by a novel series of enzymic conversions that confirm and extend the iodination data.

Scheme I. Possible Enolization Reactions Involving Dihydroxyacetone Phosphate and Glyceraldehyde 3-Phosphate.



The species involved in the enolization of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate are outlined in Scheme I, which shows the five distinct species and the six rate constants that connect them. Dihydroxyacetone phosphate may enolize between C-1 and C-2 to give the common enediol, and between C-3 and C-2 to give the enol phosphate. Both D- and L-glyceraldehyde 3-phosphate may enolize to the common enediol. Not shown in Scheme I are the hydrated forms of each of the triosephosphates, but even though these forms are significant in aqueous solution (41% for dihydroxyacetone phosphate and 96.2% for glyceraldehyde 3-phosphate: Gray and Barker, 1970; Reynolds et al., 1971), the rate constants for hydration–dehydration are approximately two orders of magnitude larger than those for enolization, and do not therefore affect the results obtained.

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Experimental Section

Materials

α-Glycerolphosphate dehydrogenase and D-glyceraldehyde-3-phosphate dehydrogenase (both from rabbit muscle) were obtained from Sigma. Each of these enzymes was freed from contaminating triosephosphate isomerase activity using the specific inactivator bromohydroxyacetone phosphate (de la Mare et al., 1972), and then dialyzed exhaustively to remove ammonium sulfate. Triosephosphate isomerase was prepared from chicken breast muscle by Mr. J. Law according to the method of Putman et al. (1972).

Dihydroxyacetone phosphate and DL-glyceraldehyde 3-phosphate were obtained from Sigma as salts of the ketal and acetal, respectively, and liberated from these precursors according to the manufacturer's instructions. For all measurements of enol percentage, and for most other kinetic measurements, the free triosephosphates were further purified as described below. L-Glyceraldehyde 3-phosphate was prepared by the enzyme-catalyzed removal of the D enantiomer using D-glyceraldehyde-3-phosphate dehydrogenase, NAD^+, and sodium arsenate, followed by chromatographic purification of the L enantiomer (see below).

All triosephosphates used were purified by chromatography on Dowex-1 (Cl^- form) ion-exchange resin. The sample (ca. 40 m*M*) was applied to the column at pH 3.8 in 0.16 m*M* HCl, and the column was washed with the equilibrating solution. A shallow pH gradient from pH 3.0 to pH 1.1 (0.16 m*M* HCl to 80 m*M* HCl) was used to elute the substrates, fractions containing which were pooled and freeze-concentrated before use.

Methods

Rate constants were determined at 30 ± 0.5°C using a Unicam SP1800 spectrophotometer. Enol percentage measurements were performed on a Durrum-Gibson stopped-flow apparatus.

Substrate Assays. Dihydroxyacetone phosphate was assayed by the addition of α-glycerolphosphate dehydrogenase (1 μl of a solution of 5 mg/ml) to a solution containing 200 m*M* triethanolamine-HCl buffer (pH 7.40), NADH (0.3 m*M*), and dihydroxyacetone phosphate (50-μl sample) in a total volume of 3.0 ml. The fall in absorbance of NADH at 340 nm was followed.

D-Glyceraldehyde 3-phosphate was assayed analogously, the assay mixture containing in addition, triosephosphate isomerase (1 μl of a solution of 5 mg/ml).

L-Glyceraldehyde 3-phosphate was assayed by measurement of the inorganic phosphate liberated after incubation for 10 min at room temperature in 1 *N* NaOH. The liberated phosphate was estimated by addition of a sample (200 μl) to a mixture of 40 m*M* ammonium molybdate (1.5 ml), 1.7 *M* sodium bisulfite (0.75 ml), and 40 m*M* hydroquinone (0.75 ml). The mixture was left for 30 min at room temperature and the absorbance at 660 nm was then measured. Standard solutions of sodium phosphate (tribasic) were used to calibrate the assay.

Rate Measurements. (For the schematic identity of the reactions *A* to *D*, see Scheme II.) (A) The uncatalyzed rate of conversion of DL-glyceraldehyde 3-phosphate into dihydroxyacetone phosphate was measured by addition of α-glycerolphosphate dehydrogenase (200 μl of a solution of 5 mg/ml) to a solution of 20 m*M* DL-glyceraldehyde 3-phosphate (3 ml), containing NADH (0.13 mg/ml), the pH of the solution being raised to pH 7.0 by addition of solid sodium bicarbonate. In this and all other reactions involving the use of coupling dehydrogenases, the reaction rate was independent of enzyme and cofactor concentration. This rules out the possibility of contaminating dehydrogenases in the coupling enzymes, and contaminating substrates in the triosephosphates.

(B) The uncatalyzed rate of conversion of dihydroxyacetone phosphate into DL-glyceraldehyde 3-phosphate was measured by the addition of D-glyceraldehyde-3-phosphate dehydrogenase (300 μl of a solution of 5 mg/ml) to a solution of 20 m*M* dihydroxyacetone phosphate (3 ml) (pH 7.0) containing NAD^+ (4 mg/ml) and sodium arsenate (2 m*M*). The rate constant from the initial rate (<0.2% conversion) of D enantiomer formation was doubled to give the rate of formation of the DL product.

(C) The *difference* in the uncatalyzed rates of conversion of L-glyceraldehyde 3-phosphate into dihydroxyacetone phosphate and into D-glyceraldehyde 3-phosphate was measured by the simultaneous addition of α-glycerolphosphate dehydrogenase (200 μl of a solution of 5 mg/ml) and D-glyceraldehyde-3-phosphate dehydrogenase (300 μl of a solution of 5 mg/ml) to a solution of 5 m*M* L-glyceraldehyde 3-phosphate (3 ml) (pH 7.0) containing NAD^+ (4 mg/ml), NADH (0.13 mg/ml), and sodium arsenate (2 m*M*).

(D) The *total* rate of conversion of L-glyceraldehyde 3-phosphate into dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate was measured by the simultaneous addition of triosephosphate isomerase (2 μl of a solution of 5 mg/ml) and D-glyceraldehyde-3-phosphate dehydrogenase (200 μl of a solution of 5 mg/ml) to a solution of 5 m*M* L-glyceraldehyde 3-phosphate (3 ml) (pH 7.0) containing NAD^+ (4 mg/ml) and sodium arsenate (2 m*M*).

Iodination reactions were performed in 50 m*M* sodium phosphate buffer (pH 7.0) containing 50 m*M* NaI. The final iodine concentration, largely present as I_3^-, was 20 μ*M* [$K = [I^-][I_2]/[I_3^-] = 1.75 \times 10^{-3}$ (Awtrey and Connick, 1951)], and the final triosephosphate concentration ranged from 60 μ*M* to 20 m*M*. The fall in absorbance of I_3^- was followed at 351 nm, and an extinction coefficient of 26,500 (Awtrey and Connick, 1951) was used. The molar extent of the observed reaction varied from 0.1 to 30% of the triosephosphate present. Purification of the triosephosphates by ion-exchange chromatography had no effect on the reaction rates.

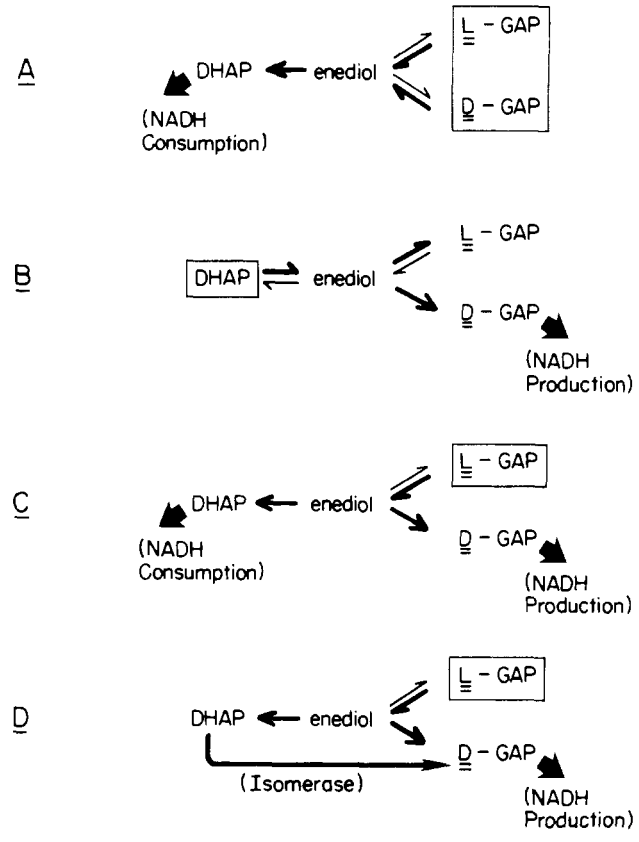
Measurement of the equilibrium enol contents of purified samples of dihydroxyacetone phosphate and DL-glyceraldehyde 3-phosphate were made in the stopped-flow apparatus, the rapid initial burst being taken as the enol content. Chromatographic purification of the triosephosphates reduced the measured bursts by less than threefold. Prior incubation of the substrate with up to 0.03% molar proportion of I_3^- (see, e.g., Bell and Smith, 1966) had no effect on the magnitude of the bursts measured subsequently after reequilibration.

Results

The use of the two dehydrogenase-catalyzed reactions for following the rates of production of either dihydroxyacetone phosphate or of D-glyceraldehyde 3-phosphate is shown in Scheme II.

In A, glycerolphosphate dehydrogenase and NADH rapidly reduce dihydroxyacetone phosphate to α-glycerol phosphate as dihydroxyacetone phosphate is spontaneously formed from DL-glyceraldehyde 3-phosphate. The dehydrogenase must, of course, be completely free from any traces

Scheme II. Experiments to Determine the Uncatalyzed Enolization Rates of Dihydroxyacetone Phosphate (DHAP) and Glyceraldehyde 3-Phosphate (GAP).[a]



[a] The starting materials are enclosed in boxes, and the presence of coupling enzymes to remove products irreversibly is indicated by heavy arrows. The direction of flux of material is indicated by the heavier lines (⇌).

of triosephosphate isomerase activity, and this was achieved using bromohydroxyacetone phosphate, a specific inactivator of the isomerase (de la Mare et al., 1972). The equilibrium constant for the dehydrogenase reaction is 10^{12} in favor of α-glycerol phosphate (Beisenhertz, 1955).

In **B**, the spontaneous isomerization of dihydroxyacetone phosphate to D- and L-glyceraldehyde 3-phosphate is followed by monitoring the oxidation of D-glyceraldehyde 3-phosphate using isomerase-free D-glyceraldehyde-3-phosphate dehydrogenase, NAD^+, and arsenate (Beisenhertz, 1955). In this reaction, 3-phosphoglycerate is produced irreversibly.

These two experiments, A and B, yield the spontaneous rates of interconversion of the two triosephosphates, which may be checked with the known equilibrium constant for this reaction of 22 (Veech et al., 1969). The overall equilibrium constant is equal to $k_{-1}k_{-2}/k_1k_2$ (Scheme I), which is given by the ratio of the composite rate constants k_A/k_B (Table I). The value of k_A/k_B from Table I yields 21 ± 9, which is in good agreement with the value of this parameter determined directly.

The experiments C and D of Scheme II are designed to determine the partition ratio (k_2/k_{-1}) of the enediol intermediate in the interconversion of dihydroxyacetone phosphate and D- or L-glyceraldehyde 3-phosphate. The enediol formed from L-glyceraldehyde phosphate can partition in three ways: (a) back to L-glyceraldehyde phosphate, (b) to D-glyceraldehyde-3-phosphate (which in both experiments C and D is oxidized rapidly and irreversibly by D-glyceraldehyde-3-phosphate dehydrogenase and NAD^+, with the concomitant production of NADH), and (c) to dihydroxyacetone phosphate.

In experiment C, dihydroxyacetone phosphate is rapidly and irreversibly reduced to glycerol phosphate with the *consumption* of NADH, and D-glyceraldehyde phosphate is oxidized rapidly and irreversibly with the *production* of NADH. The initial net rate of production (or consumption) of NADH monitored at 340 nm therefore measures the *difference* in the rates of production of the two immediate products from the enediol: dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate.

In experiment D, such dihydroxyacetone phosphate as is formed from the enediol is rapidly equilibrated stereospecifically with D-glyceraldehyde phosphate by the enzyme triosephosphate isomerase via a nondissociable enzyme-bound enediol intermediate (Rieder and Rose, 1959), and is rapidly removed from consideration through D-glyceraldehyde phosphate (see Scheme II). This experiment therefore measures the *sum* of the rates of production of the two immediate products from the enediol: dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate.

To complete the analysis, the enolization rates of dihydroxyacetone phosphate and of DL-glyceraldehyde 3-phosphate were measured by iodination of the enediol by I_2 (I_3^- is thought not to contribute directly to iodination: Dubois and Toullec, 1971). The consumption of I_2 is measured from the fall in absorbance of I_3^- (with which I_2 is in rapid equilibrium) at 351 nm. The use of a rapid mixing technique allows the burst of I_2 consumption due to the equilibrium percentage of enediol, to be measured, providing a measure (albeit approximate: see the Discussion) of the equilibrium enediol content.

From k_C/k_A, we obtain the partition ratio of the enediol intermediate, k_2/k_{-1} (Scheme I) of 3.6 ± 1.2. That is, the common enediol collapses some four times faster to glyceraldehyde 3-phosphate than to dihydroxyacetone phosphate. Substitution of this partition ratio k_2/k_{-1} into k_A, yields an approximate value for k_{-2} of $3.5 \pm 1.5 \times 10^{-5}$ sec^{-1}. This compares very well with the value of k_{-2} determined directly by iodination of DL-glyceraldehyde phosphate, of $3.0 \pm 0.2 \times 10^{-5}$ sec^{-1} (Table I), and gives some confidence in the validity of each of the very different ways of obtaining the rate data.

Since the overall equilibrium constant is $k_{-1}k_{-2}/k_1k_2$ and is accurately known to be 22 under the conditions of our experiments (Veech et al., 1969), and we know k_2/k_{-1} and k_{-2}, k_1 may be derived, and is $3.8 \pm 1.3 \times 10^{-7}$ sec^{-1}.

Finally, in order to obtain k_2 and k_{-1} (the *ratio*, k_2/k_{-1}, is derived above) we have to make use of the proportion of enediol at equilibrium with DL-glyceraldehyde 3-phosphate. Such determinations are notoriously unreliable, and must properly be considered as upper limits, since the presence of minute traces of readily iodinated impurities may seriously affect the observed value. The value obtained for DL-glyceraldehyde phosphate was 0.012 ± 0.002%: i.e., $k_{-2}/k_2 = 0.00024$. We are encouraged to put some faith in this value since (a) purification of the glyceraldehyde phosphate by column chromatography with gradient elution decreased the observed percentage of enol at equilibrium by less than threefold, and (b) reaction of the enediol with an excess of I_2, followed by a second determination of the burst of I_2 consumption after equilibrium had been reestablished (as

Table I: Values of Rate Constants for the Uncatalyzed Interconversion of Dihydroxyacetone Phosphate and Glyceraldehyde 3-Phosphate.[a]

Reaction	Scheme II	Rate Constant (from Scheme I)	Experimental Value (sec^{-1})
DL-Glyceraldehyde 3-phosphate to dihydroxyacetone phosphate	A	$k_A = k_{-1}k_{-2}/(2k_2 + k_{-1})$	$4.3 \pm 0.7 \times 10^{-6}$
Dihydroxyacetone phosphate to D- and L-glyceraldehyde 3-phosphate	B	$k_B = k_1k_2/(2k_2 + k_{-1})$	$2.1 \pm 0.7 \times 10^{-7}$
L-Glyceraldehyde phosphate to D-glyceraldehyde phosphate *less* that to dihydroxyacetone phosphate	C	$k_C = k_{-2}(k_2 - k_{-1})/(2k_2 + k_{-1})$	$1.1 \pm 0.1 \times 10^{-5}$
L-Glyceraldehyde phosphate to D-glyceraldehyde phosphate *plus* that to dihydroxyacetone phosphate	D	$k_D = k_{-2}(k_2 + k_{-1})/(2k_2 + k_{-1})$	$1.9 \pm 0.2 \times 10^{-5}$
DL-Glyceraldehyde phosphate to the enediol (captured by I_2)		k_{-2}	$3.0 \pm 0.2 \times 10^{-5}$
Dihydroxyacetone phosphate to the two enediols (captured by I_2)		$k_1 + k_3$	$7.4 \pm 1.5 \times 10^{-6}$

[a] All experiments at 30°C, pH 7.0. Each of the values for reactions A–D are the mean of three determinations, and each of the values for the iodination reactions are the mean of eight determinations.

recommended by Bell and Smith, (1966)), had no effect on the observed value. Using $k_{-2}/k_2 = 0.00024$, therefore, yields values of k_2 of 0.12 ± 0.03 sec^{-1} and k_{-1} of 0.035 ± 0.02 sec^{-1}. For the reasons outlined above, these must be considered as lower limits. [It may be noted that the determination of the equilibrium enol content of dihydroxyacetone phosphate does not provide useful information, since this material may enolize in two ways; see Scheme I.] The rate constants required are collected in Table II. Also in Table II are presented the values of the rate constants pertaining to the enolization of the carbonyl forms of the triosephosphates. Assuming that the hydrated forms do not directly form the enediol by β elimination of water, and that enediol only arises from protonation–deprotonation of the free carbonyl forms of the substrates (though it is not clear that this point has ever been satisfactorily proved), the rate constants may be corrected for the fact that dihydroxyacetone phosphate is 41% hydrated and glyceraldehyde 3-phosphate is 96.2% hydrated, under the conditions used (Reynolds et al., 1971; Trentham et al., 1969; Gray and Barker, 1970).

Table II: Derived Rate Constants for the Enolization of Dihydroxyacetone Phosphate and Glyceraldehyde Phosphate.

Rate Constant[a]	Value (sec^{-1})	Value Corrected for Hydration[d] (sec^{-1})
k_1	$3.8 \pm 1.3 \times 10^{-7}$	6×10^{-7}
k_{-1}[b]	$\geq 0.035 \pm 0.02$	≥ 0.035
k_2[b]	$\geq 0.12 \pm 0.03$	≥ 0.12
k_{-2}	$3.0 \pm 0.2 \times 10^{-5}$	8×10^{-4}
K_{eq}[c]	22 ± 0.25	330

[a] See Scheme I. [b] The *ratio* of these constants, k_2/k_{-1}, is known with reasonable accuracy, but the absolute values depend, of course, on the far less reliable measurement of the equilibrium enediol content (see the text). [c] Veech et al (1969). [d] Corrected for the fact that both of the triosephosphates are substantially hydrated in solution, and assuming that enolization occurs on the free carbonyl forms (see the text).

Discussion

The experiments described have employed the techniques of the coupled enzyme assay, to extend and confirm the more classical iodination method for the determination of enolization rates and equilibria. Although our method cannot be of very general utility, in this and other cases where the uncatalyzed rate of a biochemical transformation is under scrutiny, useful new information may be obtained. Thus it has proved possible to feed into the system the actual reaction intermediate (the enediol), using a precursor (in our case, L-glyceraldehyde 3-phosphate) that is not itself affected by the methods of assay. In this way, the partitioning rate ratio of the enediol intermediate has been obtained.

Another problem that has been solved by the use of the enzyme assay technique is the determination of the enolization rate of dihydroxyacetone phosphate. This molecule can enolize in two ways, only one of which may lead to DL-glyceraldehyde 3-phosphate (see Scheme I). Iodination, and other chemical methods, clearly give only the sum of the two enolization rates. Further, physical methods such as the monitoring of ^2H exchange by nuclear magnetic resonance (NMR) are of no use since extensive hydrolysis of the substrate occurs in the necessary time span of an NMR experiment. Using glyceraldehyde-3-phosphate dehydrogenase, however, only the enolization of dihydroxyacetone phos-

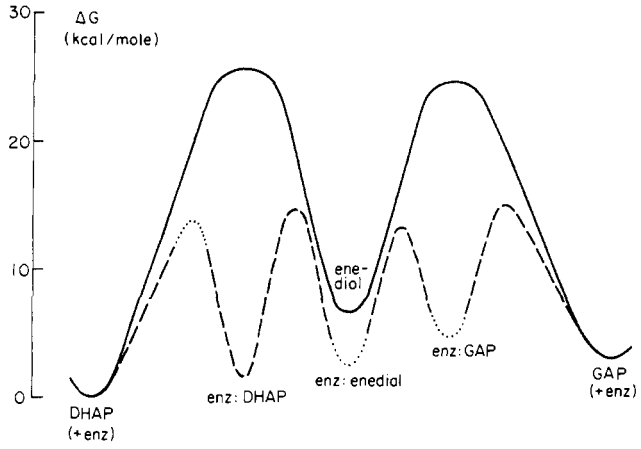


FIGURE 1: Representation of the rate constants for the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. The interconversion of the unhydrated forms of the substrates is shown: (—) uncatalyzed reaction (data from this paper); (- - -) reaction catalyzed by triosephosphate isomerase (data from W. J. Albery and J. R. Knowles, unpublished work). Dotted lines relate to parts of the free energy profile that are less well defined. A standard state of 40 μM is taken for the enzyme-catalyzed reaction, this being the concentration of triosephosphates in vivo (Williamson, 1964).

phate between C-1 and C-2 to give the "common" enediol is detected.

A pictorial representation of the rate constants for the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate for both the uncatalyzed (pH 7.0) and the triosephosphate isomerase catalyzed processes is given in Figure 1. It is evident that the enzyme has reached the end of its evolutionary development, since the highest energy barrier for the reaction is that of the diffusion to the enzyme of the thermodynamically less stable substrate, D-glyceraldehyde 3-phosphate, with a rate constant of 4×10^8 M^{-1} sec^{-1}. Further lowering of the free energy barriers for the covalency changes, or destabilization of any of the enzyme-bound intermediates, would have no effect on the flux of substrate, or, therefore, on the effectiveness of the isomerase as a catalyst.

It can be seen from Figure 1 that the enzyme accelerates the enolization of dihydroxyacetone phosphate by a factor of about 10^9, just to the point where this enolization is not rate limiting. Any further acceleration would be unprofitable in terms of the efficiency of the interconversion. It is also of interest to note that the transition states for the catalyzed reaction have been stabilized by some 7 kcal/mol more than has the enediol intermediate. This is in gratifying agreement with the view expressed by Pauling (1948) and subsequently by Wolfenden (1972) and Lienhard (1973), that a major function of enzymes is to provide stabilization for the transition states of the chemical reactions that they mediate.

Acknowledgment

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