Phosphoglucose Isomerase. II. Influence of *p*H on Kinetic Parameters*

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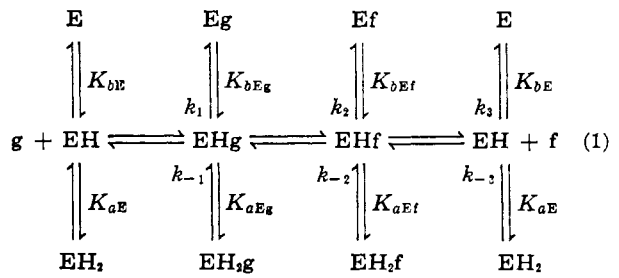
Detailed kinetic studies of mammary gland phosphoglucose isomerase have been made in both reaction directions. The influence of hydrogen ion concentration on Michaelis constants and maximum initial velocities was studied in 0.1 ionic strength Tris-acetate buffer at 30°. Kinetic data are interpreted in terms of a simple mechanism allowing for the ionization of the enzyme and the enzyme-substrate complexes. Values for the ionization constants of the enzyme and enzyme-substrate complexes have been calculated using a digital computer. The values of the "*p*H-independent" kinetic constants have also been calculated. The relationship between the kinetic parameters and the over-all equilibrium constant has been shown to be obeyed from *p*H 5.7 to 9.5.

Phosphoglucose isomerase catalyzes the interconversion of glucose-6-phosphate and fructose-6-phosphate.[1] Evidence to date indicates that this enzyme does not contain an endogenous coenzyme or require added cofactors in order to manifest its activity (Topper, 1961). Thus, the reaction catalyzed by PGI consists of the interaction of the enzyme with a single substrate to produce a single product and is representative of the simplest systems available for kinetic study. In addition, since the equilibrium concentration of G6P is only three times that of F6P at neutral *p*H values (Kahana *et al.*, 1960; *vide infra*), kinetic studies of both the forward and reverse reactions are favorable. In the present investigation, the variation of the maximum initial velocities and Michaelis constants with respect to hydrogen ion concentration for the forward and reverse reactions has been determined in the *p*H range 5.7–9.5.



The mechanism of the most general type discussed by Waley (1953) and refined by Frieden and Alberty (1955) to explain the effect of *p*H on fumarase activity has been assumed in the present studies for the interpretation of experimental results. This mechanism is reproduced and discussed briefly here in the interest of explicitness and expediency.

In this mechanism, g and f represent the anions of G6P and F6P, respectively. The active center of the enzyme E is considered to include two dissociable acid groups having acid ionization constant K_{aE} and K_{bE} (Reiner, 1959). These two acid groups are also considered to be capable of dissociation when the enzyme is present as its substrate complexes. The ionization constants are designated K_{aEg} and K_{bEg} for the enzyme-G6P complex and K_{aEf} and K_{bEf} for the enzyme-F6P complex, as indicated in the mechanism above. The assumption is made that only the monoprotonated form of the active center in the enzyme-substrate complexes yields products.

The steady-state treatment for the case that the hydrogen ion equilibria are adjusted rapidly and the substrate concentration is much greater than the total enzyme concentration (expressed in enzymically active sites per mole per liter) results in equations relating the dependence of the maximum initial velocity V and Michaelis constant K_m to *p*H (Alberty and Massey, 1954; Frieden and Alberty, 1955):

$$V_g = \frac{V_g'}{1 + (H^+)/K_{aEg}' + K_{bEg}'/(H^+)} \quad (2)$$

and

$$K_g = K_g' \frac{1 + (H^+)/K_{aE} + K_{bE}/(H^+)}{1 + (H^+)/K_{aEg}' + K_{bEg}'/(H^+)} \quad (3)$$

where V_g' and K_g' are the so-called "*p*H-independent" kinetic parameters. The equations presented here apply to the kinetic parameters in the forward reaction. Similar equations are derived for the reverse reaction. The constants K_{aEg}' and K_{bEg}' are apparent ionization constants while K_{aE} and K_{bE} are intrinsic ionization constants (Alberty, 1956). Equation (2) represents the bell-shaped curve of V vs. *p*H which is frequently obtained experimentally.

In addition, advantage has been taken of the fact that the *p*H dependence of the denominators of the equations for the maximum initial velocities and Michaelis constants are the same (Alberty and Massey, 1954) so that

$$\frac{V_g}{K_g} = \frac{V_g'/K_g'}{1 + (H^+)/K_{aE} + K_{bE}/(H^+)} \quad (4)$$

Thus, a plot of V_g/K_g will also be bell-shaped.

METHODS

Enzyme.—Phosphoglucose isomerase was isolated from bovine mammary gland according to the procedure of Baich *et al.* (1960). The enzyme, in distilled water, was frozen in 0.5-ml aliquots at a concentration of approximately 5 units/ml. A unit of PGI activity is defined as the amount of enzyme forming 1.0 μ-mole of F6P/min under the conditions described by Baich *et al.* (1960).

Reagents.—Sodium glucose-6-phosphate of 98–100% purity and tris(hydroxymethyl)aminomethane (Sigma 121) were purchased from the Sigma Chemical Company. Assays of the G6P with G6P dehydrogenase

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[1] Abbreviations used: PGI, phosphoglucose isomerase; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; Tris, tris-(hydroxymethyl)aminomethane; TPN^+, triphosphopyridine nucleotide.

and TPN^+ gave results in agreement with the stated purity (assuming the molar absorbancy index of reduced TPN^+ to be 6.22×10^3 liters $mole^{-1}$ cm^{-1}).

All other chemicals were reagent grade and used without further treatment.

Preparation of Fructose-6-Phosphate.—Analyses of commercial preparations of F6P from several sources using G6P dehydrogenase, TPN^+, and PGI showed that these preparations were far from pure. These findings agree with those reported by Kahana *et al.* (1960). Attempts to prepare highly purified F6P from this material by the method of Khym and Cohn (1953) were made and found to be technically difficult to apply on larger quantities of material. Therefore, F6P was prepared by the following procedure from G6P of 98–100% purity using the highly purified isomerase.

A mixture of 3.85 mmoles of G6P, 0.4 mmole of Tris adjusted to *p*H 7.8 with acetic acid, and 2.0 units of the enzyme in 40 ml total volume was allowed to stand at room temperature for 6 hours. At the end of this time, the reaction was stopped by the addition of 60% perchloric acid to *p*H $\leqslant 1$. Addition of a 3-fold excess (with respect to G6P) of $Ba(OH)_2$ raised the *p*H to approximately 6.0, resulting in the precipitation of the barium salt of G6P. Standing overnight at 4° facilitated the precipitation. The Ba·G6P $7 \cdot H_2O$ precipitate was removed from the F6P solution by filtration, treated with Dowex 50 (H^+ form, 50–100 mesh) and the resulting solution neutralized with 1 N NaOH after removal of the resin by filtration. The G6P recovered in this manner was twice more converted to equilibrium mixtures of G6P and F6P with the enzyme and the F6P recovered in the filtrates.

The F6P filtrates were combined and the volume reduced *in vacuo* to 10 ml. Seed crystals of Ba·G6P $7 \cdot H_2O$ were added and the solution stored for 3–4 weeks at 4°. A further precipitation of the barium salt occurred and was removed by filtration. The volume of the filtrate was increased to 25 ml to facilitate handling, Dowex 50 (H^+ form, 50–100 mesh) sufficient to remove all cations was added, and the solvent and volatile acids were removed *in vacuo*. Ten ml of distilled water was added to the highly viscous and deliquescent product and the solution was neutralized with 1 N NaOH. A yield of 1.86 mmoles of F6P was obtained.

Assay of the product with G6P dehydrogenase, TPN^+ and isomerase indicated a purity of 97–98%.

Assay Procedure.—Enzyme activity as initial velocity was determined by measuring the rate of change of F6P concentration. Initial studies demonstrated a proportionality of reaction rate to enzyme concentration (for enzyme concentrations both above and below those actually used in the later experiments). In addition, it was found that PGI was stable, under assay conditions, over the range *p*H 5.0–9.5. This was demonstrated by incubating the enzyme in 0.1 M Tris-acetate buffers at 30° for 10 minutes and assaying aliquots of the enzyme at standard conditions (0.1 M Tris-acetate buffer, *p*H 8.0, 10^{-3} M G6P, 30°). The enzyme was not stable under these conditions at *p*H 4.0. All *p*H measurements were performed electrometrically using a Beckman Model G *p*H meter.

A scaled-up method for fructose determination (Roe, 1934) was used for these experiments in preference to linked-assay systems (Kahana *et al.*, 1960). This method, although more limited with respect to ranges of substrate concentration employable, is considerably simpler, especially in the direction G6P → F6P, and involves less experimental manipulation, even if all necessary components for a linked-assay system were available in pure form. Moreover, the necessity for ascertaining the influence of *p*H on the linking enzymes and the inhibitory effect of products of such systems on PGI was avoided.

The substrates, at appropriate concentrations (*vide infra*), and Tris-acetate buffer at various *p*H (final ionic strength = 0.1 with respect to acetate except at *p*H > 9.0, where $\mu = 0.05$) in a total volume of 4 ml were equilibrated in a water bath at 30 ± 0.1°. Twenty × 150-mm test tubes served as reaction vessels. The reactions were started by the addition of 0.01 ml of enzyme on a lucite holder (0.02 ml of enzyme was used at the extremes of *p*H). The reactions were stopped after 1 minute by the addition of 1.0 ml of concentrated HCl from a blowout pipet. (It was found that the reaction rate was linear for times greater than 1 minute at all substrate concentrations used: thus, initial velocities were measured.) All concentrations of substrate were run in triplicate plus a blank. Five or six substrate concentrations were used at each *p*H, including the extremes of the concentrations used (10^{-4}–10^{-3} M for G6P and 10^{-5} to 5×10^{-5} M for F6P). The *p*H of incubation mixtures was checked after mixing of buffer and substrate, but before addition of enzyme. In addition, a reference assay in triplicate having a G6P concentration of 10^{-3} M and *p*H 8.0 was included with each run. This was done in order to facilitate corrections, if any, for changes in enzyme activity.

After all reactions were completed, 4.0 ml. of 0.1% resorcinol in 95% ethanol and 11.0 ml of concentrated HCl were added to each tube from burets, the contents mixed, and the color due to F6P developed by incubation at 80° for 10 minutes (total elapsed time). After cooling, the optical densities were read at 530 mμ using a Beckman DU spectrophotometer and 10-cm cells.

Fructose-6-phosphate prepared by the above-described procedure gave 95% as much color as fructose by the method of Roe (1934). Consequently, all experimental data were corrected for this difference.

The concentration range quoted for G6P encompassed the K_m value at all values of *p*H tested. In the case of F6P, this consideration is true only at *p*H values less than 8.0. It was not possible to include concentrations in the range of the Michaelis constant values at higher *p*H due to experimental limitations. Furthermore, the concentration ranges used in these experiments represent the minimum concentrations employable under the experimental conditions described herein.

Kinetic Constants and Ionization Constant Determinations.—The maximum initial velocities (V), Michaelis constants (K_m), and the ratios of V to K_m were computed using a digital computer programmed for a least squares fit to a hyperbola. Values of the ionization constants were obtained similarly using a program for a least squares fit to a bell-shaped curve. The latter computations used the output from the least squares fit to a hyperbola program with weighting factors in a manner similar to that described by Wilkinson (1961). This procedure also yielded values for the standard errors of the kinetic parameters and ionization constants as well as values of the "*p*H-independent" kinetic parameters.

Results

The molecular weight of mammary gland PGI has been redetermined using the sedimentation equilibrium method and interference optics.[1] The molec-

[1] Details to be reported elsewhere.

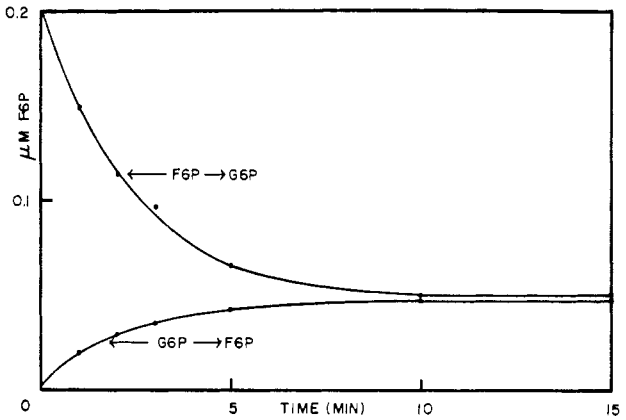

FIG. 1.—The determination of the equilibrium composition for the phosphoglucose isomerase–catalyzed interconversion of G6P and F6P. Equilibrium was approached from both sides under identical conditions yielding a value of 0.32 for the thermodynamic equilibrium constant at 30°.

ular weight was calculated to be 125,000 instead of 48,000 as previously reported (Baich *et al.*, 1960).

The molecular activity[3] (formerly termed the turnover number, Thompson, 1962) of the mammary gland enzyme was determined for the conditions prevailing in the standard assay; viz., Tris-acetate buffer, *p*H 8.0 and 0.1 ionic strength, 30°, and 10^{-3} M G6P. The value for the molecular activity was calculated to be 4.8×10^4 moles G6P/min/mole enzyme, assuming a molecular weight of 125,000.

The equilibrium constant for the reaction G6P $\rightleftarrows$ F6P as catalyzed by mammary gland PGI was determined by measuring the equilibrium concentration of F6P at *p*H 8.0 and 30° in Tris-acetate buffer of 0.1 ionic strength. Equilibrium was approached from both directions using an initial substrate concentration of 5×10^{-5} M (Fig. 1). Agreement to within ± 2% for the composition of the equilibrium mixture was found in both cases. The equilibrium constant for the reaction as written above was calculated to be 0.32. This value is in very close agreement with the value found for rabbit muscle PGI by Kahana *et al.*, (1960). In addition, the apparent equilibrium constant calculated from kinetic parameters, viz., $K_{app} = V_g K_f / V_f K_g$ (Haldane, 1930), agreed satisfactorily in the *p*H range 5.7–9.5 (*vide infra*) with the value for the thermodynamic equilibrium constant reported here.

The variation of the maximum initial velocities, expressed as molecular activities, with respect to *p*H for the forward and reverse reactions is shown in Figure 2(A) and 2(B). As predicted by equation (2), the graphs of V_g and V_f vs. *p*H were found to be symmetrical and bell-shaped. The values for the apparent ionization constants, pK_a' and pK_b', of the enzyme-substrate complexes are 5.96 ± 0.02 and 9.48 ± 0.07, respectively, for the PGI-G6P complex, and 6.82 ± 0.03 and 9.83 ± 0.30, respectively, for the PGI-F6P complex.

The mechanism represented in equation (1) requires that the ionization constants for the free enzyme have the same value when obtained from data on the forward and reverse reactions. Furthermore, as a consequence of the relationship of the apparent equilibrium constant, K_{app}, to the kinetic parameters (Haldane, 1930), plots of V_g/K_g vs. *p*H and $K_{eq}V_f/K_f$ vs. *p*H should be superimposable. Figure 3 shows that these conditions are satisfied by the experimental

[3] The molecular activity was calculated assuming one active site per enzyme molecule.

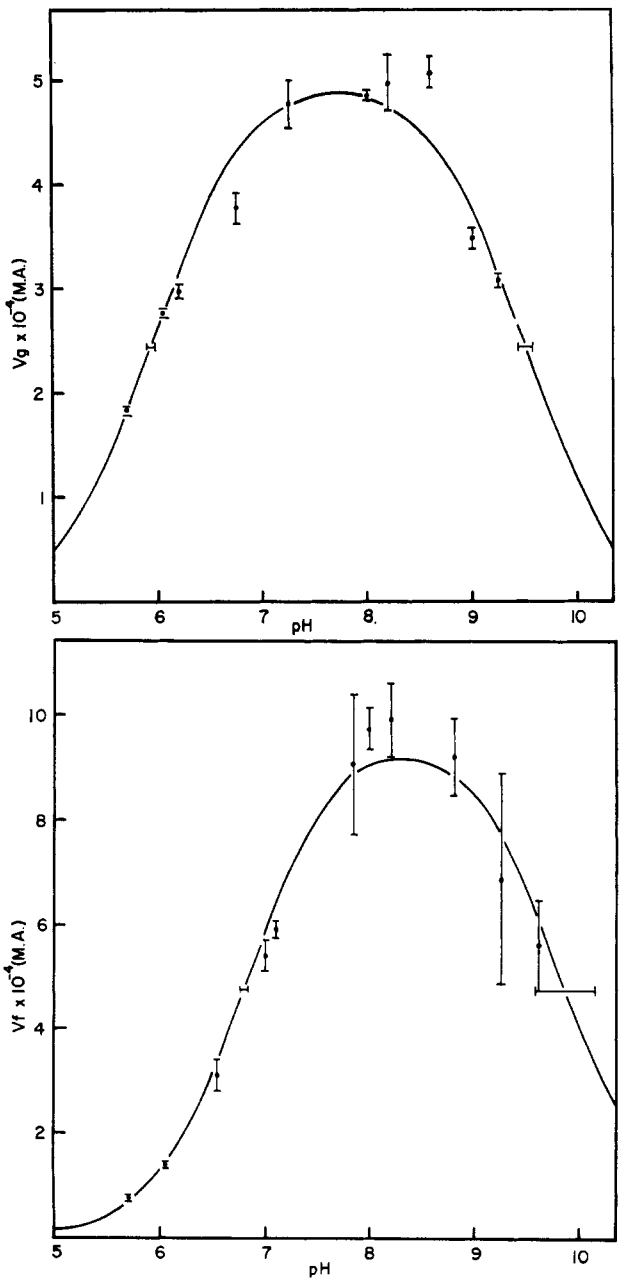

FIG. 2.—The effect of *p*H on the maximum initial velocity for (A) G6P and (B) F6P in Tris-acetate buffer (μ = 0.1) at 30°. The values for the individual points were obtained with a digital computer programmed for a least squares fit to a hyperbola. The vertical bars indicate the magnitudes of the standard errors of the maximum initial velocities and the horizontal bars the standard errors of the ionization constants. The latter were calculated with a computer programmed for a least squares fit to a bell-shaped curve (equation 2) using weighted output data from the hyperbola program. The solid lines represent the theoretical curves obtained using equation (2) and the pK' and V' values given in the text.

data which also gives the symmetrical, bell-shaped curve predicted by equation (4). Figure 3 also shows that there is good agreement between the thermodynamic equilibrium constant and the value of the equilibrium constant calculated from kinetic parameters (K_{app}) throughout the *p*H range investigated, indicating that substrate inhibition or activation was not apparent at the concentrations used in these experiments. The values for pK_a and pK_b for the free enzyme were computed to be 5.96 ± 0.06 and 9.11 ± 0.05, respectively.

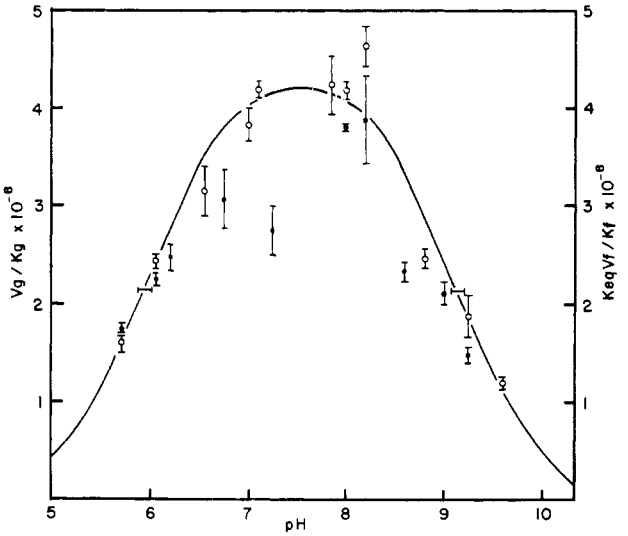

FIG. 3.—Plot of V_g/K_k (closed circles) and $K_{eq}V_f/K_f$ (open circles) versus pH. The values for the individual points were obtained with a digital computer. Vertical bars indicate the magnitudes of the standard errors of the V/K values, and horizontal bars represent the standard errors of the ionization constants. The latter were calculated via a least squares fit to a bell-shaped curve program (equation 2) using weighted output data from the least squares fit to a hyperbola program. The solid lines represent the theoretical curve calculated using equation (4), the pK values given in the text and, V' and K' values of Table I.

The variation of the Michaelis constants with respect to pH for the forward and reverse reactions is shown in Figure 4(A)[4] and 4(B).

The values for the pH-independent maximum initial velocities, V_g' and V_f', and those for the pH-independent Michaelis constants, K_g' and K_f', are listed in Table I.

The enzyme had no detectable activity on free glucose.

Discussion

The scheme depicted in mechanism 1 seems to be the simplest one consistent with all of the experimental facts. A simpler mechanism, involving only one species of enzyme-substrate intermediate, may be excluded by the experimental results obtained. Such a mechanism would require that $pK'_{aEg} = pK'_{aEf}$, and $pK'_{bEg} = pK'_{bEf}$, a condition not satisfied by our data.

Figures 2A and 2B show reasonably good agreement between the maximum initial velocity data in both reaction directions and equation (2), which is represented by continuous lines. The presence of hexose phosphate partially of an ionic form which may not function as a substrate does not influence the value of V since the latter was obtained under conditions which were zero order with respect to substrate (by extrapola-

[4] The K_g value reported in preliminary studies (Baich *et al.*, 1960) at pH 7.3, 37°, and 0.08 ionic strength was about five times the value given in this communication because different experimental conditions were employed.

TABLE I

pH-INDEPENDENT MAXIMAL INITIAL VELOCITIES, EXPRESSED AS MOLECULAR ACTIVITIES, AND MICHAELIS CONSTANTS FOR GLUCOSE-6-PHOSPHATE AND FRUCTOSE-6-PHOSPHATE RESULTING FROM COMPUTER ANALYSES OF THE EXPERIMENTAL DATA IN FIGURES 2 AND 3

	Glucose-6-Phosphate	Fructose-6-Phosphate
V' ($\times 10^{-4}$) Molecular activity	5.02 ± 0.05	9.74 ± 0.46
K'_m, μM	120 ± 3.2	70 ± 0.48

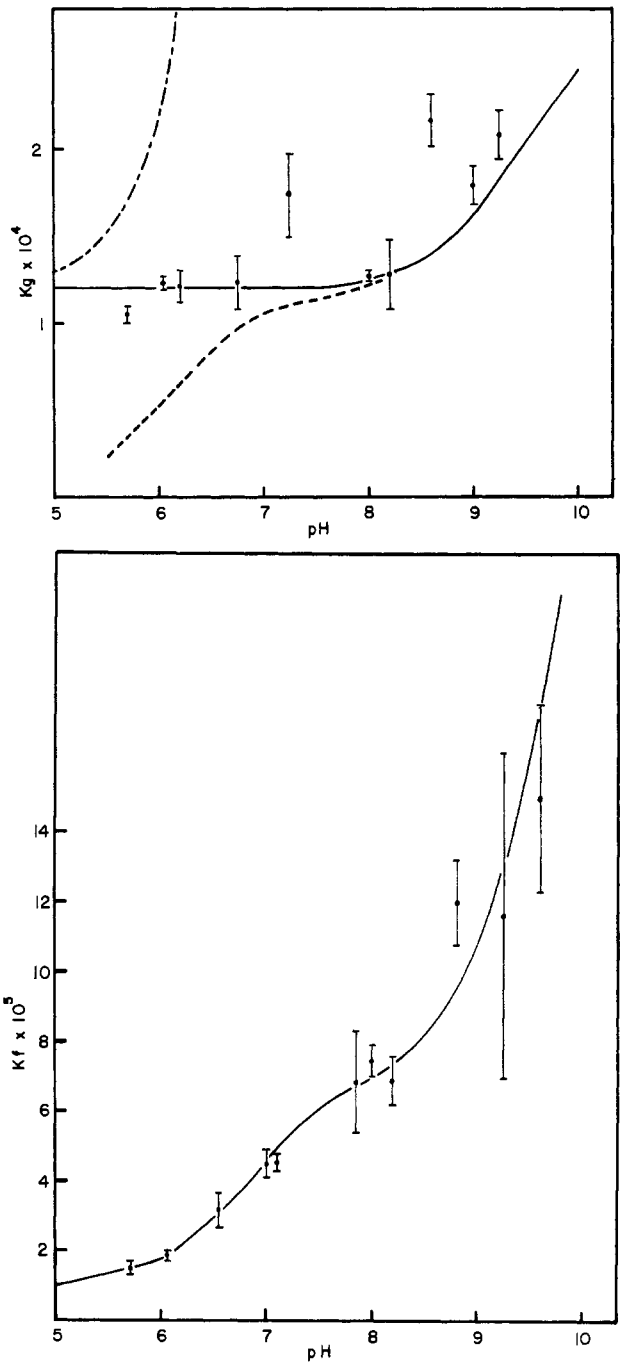

FIG. 4.—The effect of pH on the Michaelis constants for (A) G6P and (B) F6P in Tris-acetate buffer (μ = 0.1) at 30°. Values were obtained with the digital computer concurrently with those for the maximum initial velocities presented in Figure 2. The vertical bars represent the standard errors of the individual points. The continuous lines are the theoretical curves obtained using equation (3), the pK and pK' values given in the text, and K'_m values of Table I assuming that both monobasic and dibasic phosphate esters are substrates. The upper discontinuous line in (A) depicts the theoretical behavior of K_g with pH if the monobasic hexose phosphate only is the substrate. The lower discontinuous curve in (A) depicts the theoretical behavior of K_g with pH if the dibasic hexose phosphate is the only substrate for PGI.

tion). This means that variations in V with pH cannot be interpreted in terms of the substrate ionization and changes in the relative proportions of different ionic forms of the substrate. Therefore, interpretation of the bell-shaped V versus pH curve in terms of two proton dissociating groups on the enzyme is justified.[5]

If the proposed mechanism is correct, Michaelis constant values must vary with pH in a manner consistent with the participation of enzyme functional groups having pK values of about 5.9 and 9.1–9.8. Figures 4A and 4B show the agreement of the experimental data with the mechanism (represented by the continuous line). In case both ionic forms of the hexose phosphate are functional substrates, no variation in K_m with pH from that predicted by equation (3) (continuous lines, Fig. 4) will result. If the monobasic ester is the only substrate, the Michaelis constant will vary with pH as shown in the upper discontinuous curve in Figure 4A.[6] If, on the other hand, the dibasic hexose phosphate ester is the only substrate, the Michaelis constant will vary with pH as shown in the lower discontinuous curve in Figure 4A. These two theoretical curves were calculated assuming the pH independent Michaelis constant is 120 μM (Table I) and the dissociation constant for the phosphate ester is 6.1 (Meyerhof and Lohmann, 1926). Calculations involving the fructose ester Michaelis constant give similar results. Until more precise data are available in the critical pH region of 5–7, the conclusion is tentatively drawn that the fit is best to the theoretical curve for the case of both monobasic and dibasic hexose phosphate esters as substrate for PGI. Dissociation of the second proton from the hexose phosphate apparently has little or no influence on that part of the substrate molecule undergoing catalytic alteration. Moreover, the dissociation of this proton influences the magnitude and not the sign of the charge on the phosphate group. Preliminary studies indicate low but experimentally significant activity of PGI when glucose-6-sulfate is the test substrate.[7] These considerations and the fit of the experimental data to that predicted by the mechanism support the participation of both hexose phosphates as substrates for the enzyme.

The ionic state of the catalytically active form of the phosphoglucose isomerase–substrate complexes has been interpreted in terms of two acid functional groups, one of which must be protonated and the other unprotonated. This interpretation follows from a consideration of the V vs. pH curves (Fig. 2A and 2B) and K_m vs. pH curves (Fig. 4A, B). It has been pointed out (Bruice and Schmir, 1959) that an equilibrium prior to the rate-determining step may have an effect on the observed pK_a based on pH activity curves. It has also been pointed out that there is no assurance that functional groups identified through pK values calculated from activity versus pH profiles are directly involved in the catalytic mechanism (Koshland, 1960; Kosower, 1962). Until further experimental evidence warrants serious consideration of these complexities the simplest assumption implicates both groups observed in the kinetic studies of PGI as part of the

[5] An alternate mechanism in which the monobasic form of the hexose phosphate ester can function as a competitive inhibitor can be ruled out because the experimental variation of the Michaelis constant with pH does not agree with theoretical predictions. While a noncompetitive inhibition mechanism cannot be ruled out there is no strong reason for considering such a possibility.

[6] Identical influence of pH on the Michaelis and the enzyme-substrate dissociation constants is assumed as per Dixon and Webb (1958).

[7] Personal communication from Dr. F. J. Reithel.

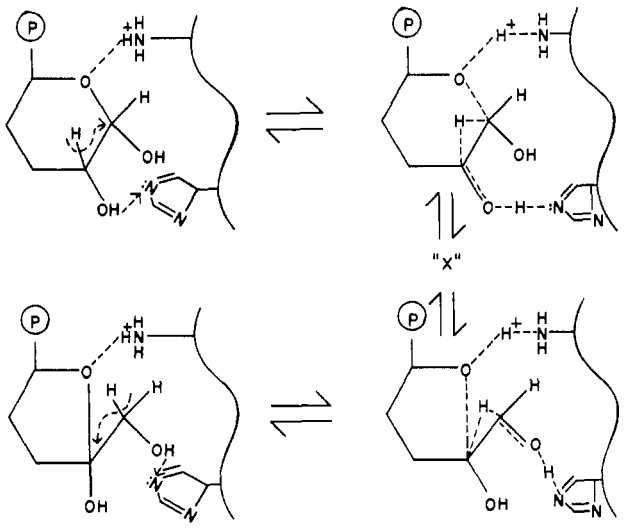

FIG. 5.—A proposed mechanism for phosphoglucose isomerase. The unprotonated functional group is depicted as attacking the hydroxyl groups on C_1 and C_2. The protonated functional group may then attack the ring oxygen of either hexose phosphate.

catalytically functional active site. The discussion to follow is based on this assumption.

Although other functional groups may be involved, the pK_a value found for the enzyme may be assumed to implicate imidazole as a constituent of the active site. A proposal that the group (or groups) having $pK \approx 5.9$ may be indicative of the participation of histidine in the catalytic site of PGI is lent further credence by the recent report of Rose and O'Connell (1961). These investigators found an intramolecular transfer of substrate hydrogen using deuterium- and tritium-labeled compounds, and from this information and theoretical considerations proposed that carboxylate or imidazole was involved in the transfer of hydrogen. Thus, there are two independent criteria supporting the participation of imidazole in the catalytic mechanism. Temperature-dependence studies of the PGI-catalyzed reaction would perhaps provide additional evidence for the participation or lack of participation of histidine in the active center of this enzyme, but the significance of the results of such studies is open to considerable question (Lindley, 1962).

If one assumes that the free-aldehyde form of the hexose is of a similar concentration in glucose and G6P solutions (approximately 0.0026%, Los *et al.*, 1956) and the free aldehyde is the true substrate form, the actual Michaelis constant would be in the range of 10^{-8}–10^{-9} M. The probability of Michaelis constants' being so small is mitigated against when it is considered that, in general, the Michaelis constants of enzymes catalyzing reactions in which reactants and products are readily dissociated have values which seldom are smaller than 10^{-6} M. Therefore, within the limits of the assumptions made, it seems reasonable to suggest that the cleavage and synthesis of the hemiacetal ring is enzyme catalyzed.

If the cyclic form of the hexose phosphate reacts directly with the enzyme, the mechanism depicted in Figure 5 can be proposed. The kinetic data suggest the participation of an unprotonated acid ($pK \approx 6$–7) and a protonated acid ($pK \approx 9.5$). The intramolecular hydride shift is consistent with the recent data of Rose and O'Connell (1961). The hydride shift mechanism has precedent in related nonenzymatic reactions involving a rearrangement of glyoxal to glycolic acid without exchange of hydrogen with D_2O (Fredenhagen

and Bonhoeffer, 1938) and a rearrangement of phenyl glyoxal to mandelic acid in D_2O without hydrogen exchange (Doering *et al.*, 1948).

Hydrogen exchange with the solvent is not accounted for in this mechanism. Such exchange may possibly occur as a consequence of substrate activation under the experimental conditions used by Rose and O'Connell. Hydrogen exchange at C-1 may be facilitated by the unilateral attack of the protonated acid on the ring oxygen of glucose in the mechanism proposed in Figure 5. This form of attack could conceivably weaken the C-H bond resulting in hydrogen exchange with the solvent.

It is obvious that much more data would be desirable in order to better understand the detailed catalytic mechanism of phosphohexose isomerase. The mechanism represented in Figure 5 is offered in the hope that it will stimulate further thinking and experimentation on this interesting reaction.

Acknowledgment

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