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The Kinetics of Phosphoglucoisomerase*

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Phosphoglucoisomerase (isomerase) is present in most tissues at very high levels of activity. Because of its strategic position in relation to alternate metabolic pathways, its kinetics are of interest. The kinetics are also of practical concern in connection with the use of glucose 6-phosphate to measure enzyme systems which produce glucose 6-phosphate, since isomerase, an ubiquitous contaminant, could distort the results if it diverted some of the product to fructose 6-phosphate.

Studies are described with partially purified isomerase from rabbit brain and muscle, and from human erythrocytes. Assay conditions were designed to prevent accumulation of the products and thereby minimize product inhibition and back reactions. The substrates consisted of enzymatically analyzed glucose-6-P and enzymatically prepared and analyzed fructose-6-P. Contaminants of chemically prepared fructose-6-P appear to have confused earlier studies and led to faulty values for the equilibrium ratio between the isomeric hexose phosphates.

The Michaelis constants have been found to be very small in both directions, but the constant for fructose-6-P is much the smaller. This results from the fact that the reaction velocity with substrate excess is approximately the same in both directions, even though equilibrium substantially favors glucose-6-P formation.

MATERIALS

Unless otherwise noted homogenates were prepared in a Waring Blendor, and all enzyme fractionations were carried out at 0 to 4°. Salt fractionations were made at a neutral pH by the addition of 1 mole of ammonium hydroxide per 50 moles of ammonium sulfate. Precipitates were removed by centrifuging at 13,000 to 16,000 × *g* for 10 to 15 minutes.

Muscle Isomerase—Rabbit muscle was homogenized with 5 volumes of water with the addition of sufficient 1 N NH_4OH to maintain the pH near 7. Material which sedimented at 8,000 × *g* in 10 minutes was discarded. The fraction soluble in 2 M ammonium sulfate and insoluble in 3 M was refractionated three times with ammonium sulfate between 1.2 and 2.3 M, 2.0 and 2.4 M, and 1.5 and 2.4 M at respective volumes of 0.34, 0.40, and 0.14 ml per g of original muscle. The yield was 28% of the original activity with 14-fold purification. Activity was 2190 moles per kg of protein per hour at 38° (measured in the direction glucose-6-P to fructose-6-P). This is comparable to the activity of a preparation described by Slein (1). The enzyme is stable for at least several weeks if kept frozen at pH 7 to 8.

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Brain Isomerase—Rabbit brain was homogenized with 9 volumes of 0.25 M phosphate buffer (pH 7.4). Insoluble fractions were removed by centrifuging and discarded both before and after addition of ammonium sulfate to a concentration of 1.5 M. The activity was precipitated at 2.6 M ammonium sulfate and then refractionated between 1.8 and 2.6 M ammonium sulfate at a volume of 2 ml per g of original brain. The purification was 11-fold with a yield of 18%. The final activity was about 2000 moles per kg of protein per hour at 38° (measured in the direction glucose-6-P to fructose-6-P).

Erythrocyte isomerase was prepared from human red blood cells washed twice with an equal volume of 1% NaCl. To 6 ml of erythrocytes were added (at 0°) 6 ml of H_2O and 12 ml of a 1:1 mixture of chloroform and methanol. After stirring 10 minutes, an additional 5 ml of water were added and the sample was centrifuged and the precipitate discarded (2). After dialyzing 2 hours against H_2O and recentrifuging, the supernatant fluid (12 ml) was treated with 15 ml of saturated ammonium sulfate. The small amount of precipitate was dissolved in 0.2 ml of H_2O and used without further purification. The activity was low, of the order of 10 moles per kg protein per hour.

Glucose-6-Phosphate Dehydrogenase—This was prepared from hog adrenal glands, which are a rich source. The assays were carried out at 25° to 28° in a volume of 1 ml with 1 mM glucose-6-P and 0.25 mM TPN^+ in 0.02 M Tris buffer (pH 8). The rate of TPNH formation (time to 0.005 or 0.01 mM) was followed directly in a fluorometer. The glands were carefully trimmed of fat, and homogenized with 9 volumes of water. The material which sedimented directly as well as that which precipitated at 1.3 M ammonium sulfate was discarded. (Throughout, the ammonium sulfate was *not* neutralized.) The enzyme was precipitated at 2.0 M ammonium sulfate with recovery of 48% of the original activity. The precipitate was dissolved, brought to a volume of 0.5 ml per g original gland, and again fractionated between 1.3 and 2.0 M ammonium sulfate with recovery of 36% of the original activity. The preparation might have been satisfactory at this point, but in order to remove hexokinase (for another purpose) it was treated for 5 or 10 minutes at pH 4.3 (acetic acid) and refractionated with ammonium sulfate. The preparation used in this study had an activity of 30 moles per kg of protein per hour at 28° and pH 8. This is equivalent to about 100 moles per kg per hour at 38° and the pH optimum (9.5). The purification was 10-fold with a yield of 5%. Isomerase activity in the direction fructose-6-P to glucose-6-P was 0.3% of dehydrogenase activity.

Brain Hexokinase—This enzyme was prepared relatively free of isomerase as a tool for making fructose-6-P directly from fructose and ATP. The preparation was an abbreviation of that of Crane and Sols (3). Rabbit brain was homogenized in 9 volumes of 0.025 M phosphate buffer (pH 7.4) and the insoluble

portion centrifuged down and washed eight times in the centrifuge with the same buffer at the same dilution. This washing only reduced the isomerase to hexokinase ratio from 7:1 to 3:1. The precipitate, suspended in a volume of 3 ml per g of original brain, was stirred at 0° for 15 minutes with sodium deoxycholate at a concentration of 0.33%. The insoluble material was centrifuged and washed six times with the above phosphate buffer with 3 to 12 ml per g of original brain. The ratio of isomerase to hexokinase activity was reduced to 0.02:1 with 60% recovery of hexokinase.

Phosphofructokinase—A rabbit was decapitated and at once chilled in ice water. The muscle was rapidly dissected and homogenized with 5 volumes of 0.05 M potassium phosphate buffer (pH 7.4). After centrifugation the supernatant fluid was quickly brought to an ammonium sulfate concentration of 2 M. The precipitate was dissolved in 0.025 M phosphate buffer (pH 7.4) (as were all subsequent precipitates) and reprecipitated in 2 M ammonium sulfate at a volume of 1.5 ml per g of original muscle. In subsequent steps the fraction was saved which precipitated between 1 and 2 M ammonium sulfate (two precipitations at 1.8 ml per g of muscle), and between 1.4 and 1.7 M ammonium sulfate (three precipitations, the first at 1.1 ml per g of muscle, the other two at 0.4 ml per g of muscle). After dialyzing for 3 hours against 0.025 M phosphate buffer (pH 7.4, 10 ml per g of muscle) insoluble material was removed by centrifuging and the fraction saved which was soluble in 1.6 M ammonium sulfate but insoluble in 1.8 M ammonium sulfate (0.5 ml per g of muscle). The activity was 1700 moles per kg of protein per hour at 30°. This is about a fourth the activity of a preparation described by Ling *et al.* (4). The purification was 60-fold with a 15% yield. Isomerase activity had been reduced to 0.2% of the phosphofructokinase activity.

Phosphopyruvate Kinase and Lactic Dehydrogenase—These were obtained together as "crystalline lactic dehydrogenase" from Sigma Chemical Company. This mixture constitutes a very convenient source of these two enzymes for the assay of ADP.[1] However, preparations were found to differ both in regard to amount of kinase and degree of contamination with certain interfering enzymes. Phosphoglucoisomerase was fortunately nearly absent, but glycerol-P dehydrogenase was present to a disturbing degree in one lot, whereas another lot was quite satisfactory (see below).

The glucose-6-P used, was the sodium salt as obtained from Sigma Chemical Company. A 3.28% solution (calculated 92 mM for disodium glucose-6-P·$3H_2O$) assayed 94.5 mM with isomerase-free glucose-6-P dehydrogenase and TPN^+. It was also found to be 92 mM in total aldose by an iodometric method. This iodometric procedure, derived from the original method of Cajori (5), may be worth recording:

A 0.4 ml sample containing 0.5 to 1.0 μmole of sugar was incubated at room temperature (25°) in a capped 3-ml tube for 2 hours with 60 μl of 0.1 N iodine in 0.12 M KI and 100 μl of 0.25 M $NaHCO_3$-0.25 M Na_2CO_3. The sample was acidified with 30 μl of 3 N HCl and titrated in ice water with 15 mM thiosulfate from a microburette with 1 drop of 1% starch at the end point.

The choice of pH (10.2) for oxidation was dictated by the fact that at more acid pH values the reaction is too slow, whereas at higher pH values iodine is rapidly converted to iodate. Because some iodate formation is unavoidable the conditions are chosen so as to consume not more than a third of the titratable iodine. The rates of oxidation of glucose and of glucose-6-P were about the same. Fructose under these conditions used only 1 or 2% as much iodine as did glucose.

Fructose-6-P—Commercial preparations of this material from several sources were far from pure. A typical sample was analyzed with resorcinol before and after treatment with glucose-6-P dehydrogenase, isomerase, and TPN^+. About 15% of the chromogenic material did not disappear (corrected for a blank with glucose-6-P identically treated). There was also present aldose (iodometric titration) equivalent to 16% of the total hexose.

In contrast, a sample of fructose-6-P kindly supplied by Dr. Robert K. Crane which he had prepared from fructose with isomerase-low brain hexokinase (6) was found to be free of chromogenic material other than fructose-6-P and to contain only 8% of total hexose as glucose-6-P.

Therefore a larger sample was prepared by incubating 3.3 mmoles of fructose, with 2 mmoles of ATP in 100 ml of 0.04 M Tris buffer (pH 8.6) containing 1 mmole of $MgCl_2$, 0.1 mmole of ethylenediaminetetraacetate, 1 mmole of phosphate, and sufficient brain hexokinase (see above) to give an activity of about 10 mmoles per liter per hour. After 165 minutes at 38° the reaction was stopped and there were found to be present 0.61 mmole of fructose-6-P and 0.09 mmole of glucose-6-P. (The assays were made with glucose-6-P dehydrogenase in the presence and in the absence of isomerase.) The nucleotides were removed with charcoal (7) and the phosphate esters were isolated together as the barium salt (6). For the experiment of Fig. 3, a similar preparation was chromatographed on Dowex 1 according to Khym and Cohn (8) and the glucose-6-P was reduced to less than 2%.

6-P-gluconate—This was a gift of synthetic material from Dr. Robert K. Crane.

ASSAY SYSTEMS

Glucose-6-P Formation—Isomerase was allowed to act at pH 8 on fructose-6-P in a 1 ml volume in a 10 × 75-mm selected Pyrex tube in a sensitive fluorometer (Farrand Optical Company). Also present were TPN^+ (0.25 mM) and sufficient glucose-6-P dehydrogenase to oxidize at least 95% of low levels of glucose-6-P in 2 minutes. The fluorescence of the TPNH produced was followed in the instrument with the use of suitable filters (9).

Since the Michaelis constant, K_s, of adrenal glucose-6-P dehydrogenase at pH 8 is about 0.03 mM, the amount of enzyme needed to meet the above requirement is such as to give a velocity with *excess* substrate (V_{max}) of at least 3 mmoles per liter per hour. This results from the fact that $V_{max} = (K_s/t$ (in hours)) ln $((S_0)/(S))$, when $(S_0) \ll K_s$. $((S_0)$ and (S) indicate substrate concentrations at time zero and time t, respectively.) Therefore, to give 95% conversion in 2 minutes, $V_{max} = (0.03/0.033)$ ln 20 = 2.7. Under these conditions after a brief lag period the steady state glucose-6-P level will be about 1% (expressed as millimoles per liter) of the *isomerase* velocity (expressed as millimoles per liter per hour). This is so, since, when $(S) \ll K_s$ (as in the present case), $v = V_{max}(S)/K_s$, or $(S) = v \times K_s/V_{max} = v \times 0.03/3$. And when the steady state is attained, the isomerase velocity is equal to the glucose-6-P dehydrogenase velocity. As an example, if 0.02 mM fructose-6-P were to be converted to glucose-6-P at a rate of 0.04 mmole per liter per hour under the above conditions, the steady state concentration of glucose-6-P would be 0.000, 4 mM or 2% of the initial fructose-6-P concentration. Since the Michaelis constant

[1] Jack L. Strominger, personal communication.

for glucose-6-P with isomerase is approximately 0.03 mmole per liter (see below), product inhibition will be negligible. Also since the maximum velocity is about equal in both directions, the back reaction will be negligible at this low glucose-6-P concentration.

Measurements were made by adding the fructose-6-P, allowing 3 minutes for any glucose-6-P initially present to be oxidized and 5 or 10 minutes for opportunity to evaluate the trace of isomerase activity in the glucose-6-P dehydrogenase. At this time isomerase was added and the velocity was determined graphically for a 4- or 5-minute interval starting 2 minutes later. Due correction was made for fructose-6-P lost while the steady state was being reached. Although inspection of the curve was used to confirm the attainment of a steady state, it may be calculated that 2 minutes is sufficient. The calculation is as follows: Let v^i be the isomerase velocity, (G) the glucose-6-P concentration, v^d the dehydrogenase velocity, and $C_2 = V^d{}_{max}/K_2$ (where K_2 is the Michaelis constant of glucose-6-P for the dehydrogenase). Steady state is attained when glucose-6-P is destroyed as fast as it is formed, *i.e.* when $d(G)/dt = 0$. The error in rate is therefore $d(G)/dt = v^i - C_2(G)$, (since $(G) \ll K_2$). Integrating, $(G) = v^i C_2(1 - e^{-C_2 t})$, from which $d(G)/dt = v^i e^{-C_2 t}$.

The fractional error in rate is $v^i e^{-C_2 t}/v^i = e^{-C_2 t}$. In the present case where $C_2 = 3/0.03 = 100$ hour^{-1}, or 1.6 min^{-1}, the error would be 19, 4, and 0.7% at 1, 2, and 3 minutes. (It may be useful to calculate that since the half-time equals $0.69/C_2$, it follows as a rule of general application that the error in rate will be less than 4% after 5 half-times for the second reaction.)

Fructose-6-P Formation—Isomerase was allowed to act on glucose-6-P in a volume of about 0.5 ml in a Beckman spectrophotometer. The fructose-6-P was trapped with phosphofructokinase and ATP (0.3 mM). The ADP resulting was measured with the system ADP + P-enolpyruvate (1 mM) → ATP + pyruvate, pyruvate + DPNH (0.13 mM) → lactate + DPN$^+$ (10). The fall in absorption at $\lambda = 340$ mμ was used to follow the reaction. Phosphofructokinase was employed at a level to give a V_{max} of 8 mmoles per liter per hour. The "lactic dehydrogenase" (see above) was used at a dilution of 50 μg of protein per ml. This was sufficent to measure at least 90% of 0.01 mM ADP in 1 minute. The complete reagent contained 3 mM K_2HPO_4 and 5 mM Mg^{++} in 0.02 M Tris.

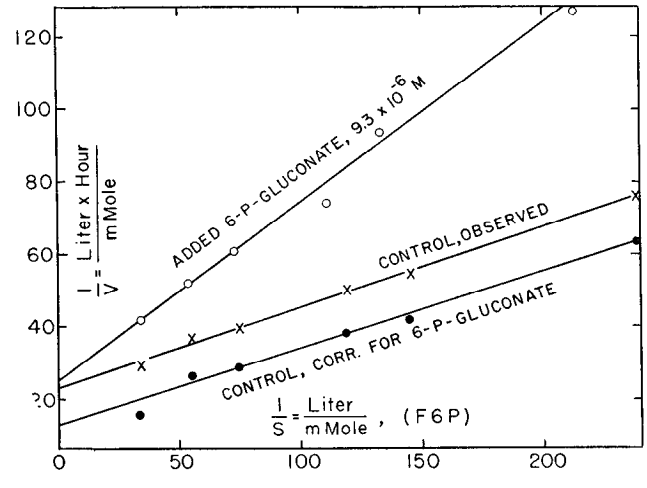

FIG. 1. Effect of substrate concentration and 6-P-gluconate on rate of conversion of fructose-6-P (F6P) to glucose-6-P by muscle isomerase. The control contained some 6-P-gluconate because of the method of assay (glucose-6-P dehydrogenase) (see text.) Measurements were made in 0.02 M Tris buffer (pH 8) with 0.25 mM TPN$^+$ and 1 mM ethylenediaminetetraacetic acid. It may seem surprising that the corrected curve is approximately parallel to the observed uncorrected curve. This results from the fact that the amount of 6-P-gluconate in the control curve is not constant, but varies approximately as the substrate varies, *i.e.* $(I)/(S)$ is a constant. Since $1/v = 1/V_{max}(1 + K_s/(S) + (I)/(S) \cdot K_s/K_i)$, all values of $1/v$ will be displaced by the constant amount $(I)K_s/V_{max}(S)K_i$.

Measurements were made by comparing rates graphically between two samples one of which had no added isomerase but was otherwise complete. After a preliminary period of 7 to 9 minutes, substrate was added to both samples, and the velocity assessed over an interval of 5 or 6 minutes after a 2 or 3 minute allowance for attainment of steady state. The enzymes used were not entirely free from aldolase, glycerol-P dehydrogenase, or triose-P isomerase, consequently formation of 1 mole of fructose-di-P might conceivably yield 3 moles of DPN$^+$. One sample of lactic dehydrogenase was unsuitable on this account. By selection of the best preparations, this difficulty was reduced to the point at which DPNH disappearance over the time interval concerned did not exceed fructose-di-P formation by more than 10%.

RESULTS

Inhibition by 6-Phosphogluconate—Parr (11) reported that 6-P-gluconate is a potent inhibitor of isomerase. 6-P-Gluconate is formed during the isomerase assay when fructose-6-P is substrate. This arises both from glucose-6-P contamination of the substrate, as well as from glucose-6-P formed during the assay. Consequently, the inhibition had to be evaluated. The activity of muscle isomerase was measured with and without the addition of 0.0093 mM 6-P-gluconate at 6 levels of substrate (fructose-6-P). The inhibition seems to be competitive (Fig. 1) (see also below). The effect of the 6-P-gluconate unavoidably present was evaluated graphically from the equation $1/v = 1/V^f_{max}(1 + K_s/(S) + K_s(I)/(S)K_i)$, where (I) and K_i represent, respectively, the concentration and dissociation constant for the inhibitor. That is, when the concentration of substrate (S) is held constant $1/v$ is a linear function of I. Consequently the effect on $1/v$ of the *added* 6-P-gluconate is proportional to the effect on $1/v$ of the 6-P-gluconate unavoidably present and therefore the values of $1/v$ in the absence of 6-P-gluconate may be calculated.[2] This was done for the experiment of Fig. 1. The corrected control line gives a K_f of 1.6×10^{-5} M from which K_i may be calculated by the above formula to have the surprisingly low value of 7×10^{-6}M. This experiment is given as an illustration. In two later but less detailed experiments concurring values of K_f for fructose 6-phosphate of 1.0×10^{-5}M, and of K_i for 6-P-gluconate of 5×10^{-6}M were obtained, and these lower values are believed to be more nearly correct. The competitive nature and magnitude of the inhibition were confirmed by holding (S) constant at 1×10^{-5}M and varying (I) from 0.4 to 5×10^{-5}M. The values obtained

[2] For example, suppose at a given substrate level there is 3×10^{-6} mole of the inhibitor, 6-P-gluconate, unavoidably present and that $1/v$ is 50 without added inhibitor and 90 with 9×10^{-6} mole of added inhibitor. The corrected value for $1/v$ would be $50 - 3/9(90 - 50) = 37$. The 6-P-gluconate concentration at any instant is equal to the TPNH concentration. The amount present midway in the assay was used in the calculation.

were in good agreement with the equation above. The same K_i was obtained with muscle and brain isomerase.

Inhibition by Other Phosphorylated Compounds—Isomerase activity was also measured in this same direction (fructose-6-P to glucose-6-P) in the presence of the ingredients which were to be needed for measuring isomerase in the opposite direction. These ingredients included ATP and P-enolpyruvate as well as P_i which probably could have been omitted. All three substances proved to be competitive inhibitors with inhibitor constants of 0.4×10^{-3}M for ATP, 1.1×10^{-3}M for P-enolpyruvate, and 1.7×10^{-3}M for P_i.

The apparent K_f with brain isomerase in the presence of ATP (0.3 mM), P-enolpyruvate (1.08 mM), and P_i (3 mM), as well as $MgCl_2$ (5 mM) was found to be 3.02×10^{-5}M (corrected for 6-P-gluconate inhibition). In a simultaneous experiment without these added materials, the K_f was observed to be 1.02×10^{-5}M. It is easily shown that if (A), (B), and (C) refer to competitive inhibitor concentrations, and K_a, K_b, and K_c indicate the respective inhibitor constants, that $(V_{max} - v/v)\ S = K_s F = K'_s$ where $F = 1 + (A)/K_a + (B)/K_b + (C)/K_c$. In this case F, calculated from the constants of the preceding paragraph, should equal 4.5 instead of 3.0 as observed. The difference is due to the Mg present.[3]

Comparison of Velocities and Michaelis Constants in Both Directions—To make comparisons as directly as possible, measurements were made with brain and muscle isomerase in both directions on the same day under conditions as nearly alike as practicable. The results (Fig. 2, Table I) are consistent with numerous other less elaborate experiments. A similar experiment is recorded for erythrocyte isomerase. The erythrocyte results are believed to be valid in the direction fructose-6-P to glucose-6-P but in the opposite direction a large blank value with the relatively crude enzyme reduced the accuracy, and the values must be considered only approximate. The summary of the results (Table I) indicates that the maximum velocities are as great in the "unfavorable" direction of fructose-6-P formation as in the favorable direction. Therefore, since equilibrium favors glucose-6-P, the Michaelis constants must be much smaller for fructose-6-P than for glucose-6-P, and this was found to be true (Table I). The apparent Michaelis constants are about 3 times the true values because of the presence of other phosphate compounds as described above. The best estimates of K_f and K_g at present appear to be 1.0 and 3.0×10^{-5}M, respectively (see below.)

Equilibrium between Fructose-6-P and Glucose-6-P—The equilibrium constant may be calculated from the Michaelis constants and maximum velocities in both directions (12). The calculated ratios approach 3:1, whereas ratios previously reported range from 1.5 (13–15) to 2.3 (16, 17). The values for the ratios have ordinarily been based on the decrease in color with resorcinol when fructose-6-P was treated with isomerase. Commerical fructose-6-P, at least in the past, has contained chromogenic material which was not fructose-6-P, as well as some preformed glucose-6-P (see above). Both of these impurities would reduce the change in chromogenic material upon addition of isomerase and give an erroneously low ratio. Low ratios were apparently confirmed by starting with glucose-6-P and measuring the increase in chromogen produced with isomerase. Here the error in calculation was due to the belief that fructose-6-P gives only 60% as much color with the resorcinol method of Roe (18) as does fructose (13–15). This value seems to be in error, and again appears to be largely the result of the poor quality of available fructose-6-P. (Slein (16) found 79% as much color with his fructose-6-P as with fructose, and observed a higher equilibrium ratio (2.3)). Dische (19) in contrast, with the use of the revised method of Roe *et al.* (20), found the same amount of color with fructose as with fructose-6-P (source not identified). In the present investigation it has been found with enzymatically prepared material (analyzed with glucose-6-P dehydrogenase and TPN^+) that fructose-6-P actually gives 5% *more* color than fructose by the revised method of Roe, and the same amount of color as fructose by the original method of Roe. This last statement is true whether the time of heating is 8 minutes (at 80°) as originally proposed or 15 minutes as some workers have recommended.

When the ratio was redetermined with enzymatically prepared fructose-6-P, values of about 3 were obtained, based on the de-

[3] In another experiment, carried out at 23° in the absence of Mg, F calculated from separately determined inhibitor constants was 4.44. The observed value was 4.02. Addition of 5 mM decreased F to 2.39. When added to the inhibitors separately, Mg had almost no effect on the inhibitor constant for P_i, doubled the apparent inhibitor constant for P-enolpyruvate, and abolished the inhibition due to ATP.

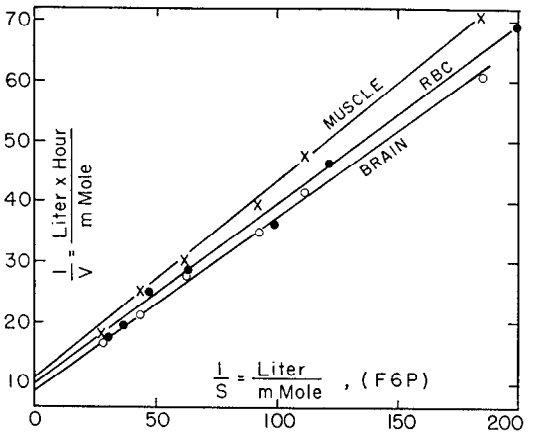

FIG. 2. Effect of substrate concentration on isomerase of rabbit brain, rabbit muscle, and human red blood cells. Measurements were made at 30° in the direction of glucose-6-P formation in the presence of 0.3 mM ATP, 1 mM P-enolpyruvate, and 3 mM P_i at pH 8 in 0.02 M Tris (see text.)

TABLE I

Isomerase kinetics

The assay conditions are described in the legend for Fig. 2. Velocities are recorded as moles per liter of stock enzyme solution per hour. Michaelis constants are given as millimoles per liter. The Michaelis constants have not been corrected for inhibition by phosphate compounds present for analytical purposes. The true constants are believed to be one third of those recorded (see text.)

Source of isomerase	F6P* → G6P*		G6P → F6P		$\frac{G6P}{F6P}$ (calculated)†
	V^f_{max}	K'_f	V^g_{max}	K'_g	
Rabbit brain	19.2	0.034	20.5	0.111	3.1
Rabbit muscle	97.5	0.031	132	0.104	2.5
Human red blood cells	0.035	0.030	0.037	(0.17)‡	

* Fructose-6-P and glucose-6-P.

† $V^f_{max} \times K_g / V^g_{max} \times K_f$ (12).

‡ This value only an approximation.

crease in chromogenicity after isomerase. An equilibrium ratio of the same order was obtained by iodometric determination of glucose-6-P before and after isomerase treatment. However, the values which are believed to be the most accurate were obtained with the aid of glucose-6-P dehydrogenase. Equilibrium mixtures of the two phosphates were obtained with isomerase at 38° starting with either glucose-6-P or enzymatically prepared fructose-6-P (Table II). The isomerase was destroyed with acid, and TPN^+ and glucose-6-P dehydrogenase were then added. After measuring the glucose-6-P (change in optical density at 340 mμ) isomerase was again added and the fructose-6-P determined. Starting with fructose-6-P an average ratio of 2.84 was obtained, starting with glucose-6-P a ratio of 2.76 was obtained (Experiment A, Table II). A somewhat higher ratio (3.06) was obtained in an experiment designed to increase the precision of fructose-6-P measurement (Experiment B, Table II). The equilibrium constant is temperature dependent with glucose-6-P favored more at lower temperature (Experiment B).

No significant change in ratio was observed when the pH was varied from 6.6 to 8.4 (resorcinol method).

Time Course of Isomerase—An experiment was conducted in which equilibrium was approached from both directions under identical conditions, as an over-all check on velocities in the two directions and the relative Michaelis constants (Fig. 3). Since the substrate levels were high (2.5 mM) the curves are insensitive to the absolute Michaelis constants. They are however sensitive to relative values of the constants and absolute velocities. The results are in reasonable accord with the initial velocity measurements observed above. The best fit between observed and calculated curves was obtained with a 3:1 ratio of K_g to K_f and a 1:1.2 ratio of V^g_{max} to V^f_{max}. The theoretical curves fit the data almost to within experimental limits although there is a suggestion of a real discrepancy in the case of fructose-6-P.

TABLE II

Equilibrium between glucose-6-P and fructose-6-P

Samples were incubated with enough rabbit muscle isomerase to give maximal velocities (at 38°) of 50, 100, and 32 mmoles per liter per hour respectively for Experiment A, glucose-6-P (G6P), Experiment A, fructose-6-P (F6P), and Experiment B. After two time intervals aliquots were acidified with HCl and were then analyzed enzymatically for G6P and F6P. See text. Values are recorded as millimoles per liter during incubation. Experiment B was conducted so as to further increase the precision of measurement. (See legend of Fig. 3.)

	Temperature	Control		Isomerase time 1*		Isomerase time 2*		Ratio,† G6P/F6P
		G6P	F6P	G6P	F6P	G6P	F6P	
Experiment A								
Glucose-6-P	38°	1.97	0.01	1.45	0.50	1.41	0.51	2.76
Fructose-6-P, preparation A	38°	0.20	1.85	1.49	0.53	1.49	0.53	2.81
Fructose-6-P, preparation B	38°	0.12	2.37	1.79	0.75	1.86	0.65	2.86
Experiment B								
Glucose-6-P	38°	2.50	0.0	1.88	0.617	1.89	0.610	3.06
Glucose-6-P	30°	2.50	0.0	1.92	0.580	1.94	0.565	3.36
Glucose-6-P	20°	2.50	0.0	1.98	0.523	1.98	0.518	3.85

* Times were 10 and 20 minutes in Experiment A, 15 and 30 minutes in Experiment B at 30° and 38° and 30 and 60 minutes in Experiment B at 20°.

† Final ratio Experiment A, average ratio Experiment B.

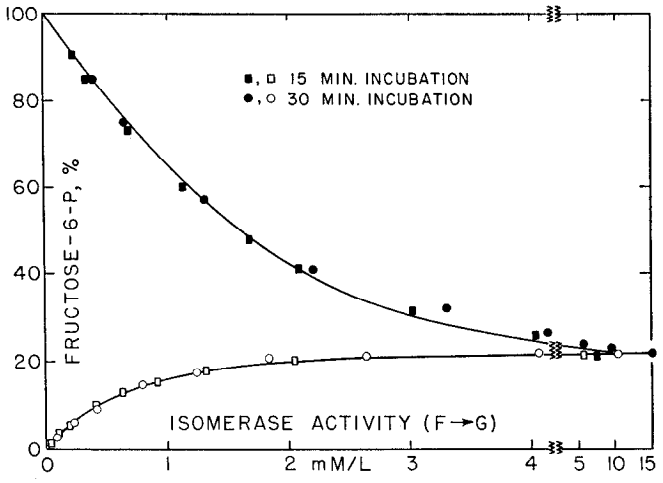

FIG. 3. Approach to equilibrium from both directions. Samples (0.2 ml) containing glucose-6-P or fructose-6-P at a concentration of 2.5 mM were incubated for 15 or 30 minutes at 30° (pH 8, 0.05 M Tris buffer, 0.05% bovine plasma albumin).

The reaction was stopped with HCl. The glucose-6-P was measured by the change in absorption at 340 mμ with added glucose-6-P dehydrogenase and TPN^+, after which isomerase was added to measure the fructose-6-P as well. To increase precision samples were diluted only enough to reduce total hexose concentration to about 0.25 mM. Optical densities over 1 were read against a solution of potassium dichromate of optical density about 1 (at 340 mμ). The isomerase was added in varying quantities and the amount is recorded as the total initial activity (initial velocity multiplied by hours of incubation) in the direction fructose to glucose. All samples were incubated simultaneously. The curves are theoretical for K_f = 0.01 mM, K_g = 0.03 mM and an equilibrium ratio of 3.6 (glucose-6-P to fructose-6-P). They were calculated from the formula:

$$V^s_{max}t = \frac{(K_p - K_s)}{(R+1)K_p} - \frac{K_pK_s(R+1) + (S_0)(K_s + K_pR)\ln\left(1 - \frac{(P)(1+R)}{(S_0)}\right)}{(R+1)^2K_p}$$

where (S) and (P) indicate substrate and product, and R the ratio of (S) to (P) (*cf.* Alberty (21)). After substituting for the constants this becomes $V^g_{max}t = -0.435(F) - (0.0065 + 0.312(G_0))\ln(1 - 4.6(F)/(G_0))$ (for the direction of fructose-6-P formation). (F) is the concentration of fructose-6-P at time t, and (G_0) is the initial concentration of glucose-6-P.

DISCUSSION

The Michaelis constants for isomerase are surprisingly low, much lower than estimates to be found in the literature. For example, Tsuboi *et al.* (2) reported a value of about 1.7×10^{-3} M for *both* glucose-6-P and fructose-6-P with red blood cell isomerase. These values appear to be at least 50- and 150-fold too high, respectively. This serves to illustrate the difficulty with measurements in a system which diverges greatly from linearity with increasing product formation. Tsuboi *et al.* apparently based rate determination on product formation after a fixed time interval with constant amount of enzyme. This would give an erroneously high result unless the enzyme activity were reduced so low as to be practically immeasurable by the method used.

An experimenter may be easily misled by the fact that product formation, as described by the equation of the legend of Fig. 3, gives an almost linear plot of $1/v$ against $1/(S)_0$ *when enzyme activity is held constant,* regardless of the degree of enzyme saturation. Consequently, completely fallacious results may be obtained with nearly perfect-appearing Lineweaver-Burk plots. In the case of isomerase a calculated K_g would be too large by about 3.2 times whatever enzyme activity happened to be used ($V^g_{max}t$). Thus, with enough enzyme and time to convert 0.5 mM glucose-6-P to fructose-6-P with substrate excess, the apparent K_g would be 1.6×10^{-3}M instead of 3×10^{-5}. To reduce the error in K_g to 10% under these conditions, it would be necessary to reduce maximum enzyme activity to 10^{-6} mole per liter, and to measure fructose-6-P formation in the concentration range of 10^{-6} to 10^{-7}M (which is scarcely feasible with the resorcinol method).

Bodansky (13) in a study of isomerase of human serum published curves for the approach to equilibrium from both directions. The curves would closely resemble those of Fig. 3 both in shape and relative velocity if the equilibrium point were merely shifted from 1.5 to 3.6. Similarly the formula of the legend to Fig. 3 gives a very close fit to the curve obtained by Bodansky for fructose-6-P formation with varied enzyme concentrations (22).

The equilibrium ratios between glucose-6-P and fructose-6-P are believed to be accurate to within 10%. The figure in Table II, Experiment B, gives a value for ΔF at 38° of −690 calories. The changes in equilibrium constant between 38° and 30°, and between 30° and 20° give values for ΔH of −2,200 and −2,400 calories, respectively. From these figures ΔS is calculated to be −5.2 calories per degree, at 38°.

The Michaelis constants although believed to be of the right order of magnitude are not as accurate as the equilibrium constants. Perhaps the most reliable value for the *ratio* of K_g to K_f is obtained from the best fit for the data of Fig. 3, *i.e.* 3.0, which agrees reasonably well with the ratios of the Michaelis constants of Table I (3.3 and 3.4). The best estimate of K_f is 1.0×10^{-5}M, which would make K_g equal to 3.0×10^{-5}M. With these values and an equilibrium ratio of 3.4 it is possible to calculate the relative values of the rate constants assuming the reaction has the simple form

$$E + G \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \times \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + F.$$

The calculation yields the *relative* values of 71 mM^{-1}, 1.13, 1, and 213 mM^{-1} for k_1, k_2, k_3, and k_4, respectively.

It is of conceivable biological significance that isomerase is inhibited by ATP, P_i, and especially by 6-P-gluconate. If, for example the glucose-6-P tissue level were 0.3 mM, *i.e.* 10 times the K_s, then ATP at a concentration of 10 mM (25 times K_i) would reduce isomerase activity by about 70%; P_i at 10 mM (6 times K_i) would reduce isomerase activity by 35%; 6-P-gluconate at 0.2 mM (40 times K_i) would reduce isomerase by nearly 80%. All three compounds together at the postulated levels could produce an 87% reduction in enzyme activity. However, because of the very high absolute activity of isomerase in tissues, it is difficult to be persuaded that inhibitions of this degree would have much significance except perhaps under special situations of intracellular compartmentation, and so forth. Possibly under some special circumstances a drop in ATP could accelerate fructose-6-P formation, or a rise in 6-P-gluconate could block isomerase enough to raise glucose-6-P levels and force more hexose into the oxidative shunt.

SUMMARY

1. A kinetic study was made of phosphoglucoisomerase from rabbit brain and skeletal muscle, and from human red blood cells. The reaction in the direction of glucose 6-phosphate formation was followed in the fluorometer by the formation of reduced triphosphopyridine nucleotide from triphosphopyridine nucleotide with a large excess of glucose 6-phosphate dehydrogenase. The reaction in the direction of fructose 6-phosphate formation was followed in the spectrophotometer by the disappearance of reduced diphosphopyridine nucleotide resulting from the following enzymatic reactions: fructose 6-phosphate + adenosine triphosphate → fructose diphosphate + adenosine diphosphate; adenosine diphosphate + phosphoenolpyruvate → adenosine triphosphate + pyruvate; pyruvate + reduced diphosphopyridine nucleotide → lactate + diphosphopyridine nucleotide. The auxiliary enzymes were added in large excess.

2. The best estimates of the Michaelis constants are believed to be $K_g = 3 \times 10^{-5}$ M, and $K_f = 1 \times 10^{-5}$M, at 30°. The maximum velocities are approximately the same in both directions.

3. The equilibrium concentrations of glucose 6-phosphate and fructose 6-phosphate were measured by enzymatic assay and the ratio was found to be considerably higher than previously reported. The difference is attributed to earlier impure (synthetic) fructose 6-phosphate. At 38°, 30°, and 20° ratios of glucose 6-phosphate to fructose 6-phosphate were found to be 3.06, 3.36, and 3.85, respectively. From these values it may be calculated that ΔF is −690 calories at 38°, that ΔH is −2300 calories, and ΔS is −5.2 calories per degree. The observed Michaelis constants and velocities in the two directions at 30° are in substantial agreement with the ratio observed at 30°.

4. The approach to equilibrium from both directions follows a course in good agreement with the theoretical curve calculated from the observed parameters.

5. Adenosine triphosphate, phosphoenolpyruvate, inorganic phosphate, and 6-phosphogluconate were all found to be competitive inhibitors of isomerase with respective inhibitor constants at 30° of 0.4, 1.1, 1.7, and 0.005×10^{-3} M.

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