Thermodynamics of Phosphate Transfer in the Phosphoglucomutase System*

Ernest J. Peck, Jr., Donald S. Kirkpatrick,† and William J. Ray, Jr.‡

ABSTRACT: The equilibrium constant for transfer of the phosphate group of phosphoglucomutase to the 1 position of glucose 6-phosphate is measured by using three independent techniques. All three techniques are generally applicable for determining equilibrium constants of enzyme–substrate reactions in Ping-Pong pathways and two of the techniques apparently have not been previously used. Essentially the same results are obtained with each procedure: [dephosphoenzyme][α-D-glucose 1,6-bisphosphate]/[phosphoenzyme][glucose 6-phosphate] $\approx 2 \times 10^{-3}$ at pH 7.4 and 2×10^{-4} at pH 8.5. Coupled with measurements of the binding of inorganic phosphate to the enzyme, these data are used to compare the free-energy change for the hydrolytic cleavage of the seryl phosphate bond of phosphoglucomutase with the corresponding free-energy change for phosphate bond breaking in alkaline phosphatase, potato phosphomonoesterase, and simple phosphate esters.

In the reaction of glucose 6-sulfate with phosphoglucomutase a single net phosphate transfer occurs to give the dephospho form of the enzyme and glucose 1-phosphate 6-sulfate; subsequent transfer of the sulfate moiety to the dephosphoenzyme is not observed. The $\Delta F°$ for hydrolysis of the 1-phosphate group of glucose 1,6-bisphosphate is −4.8 kcal.

Phosphoglucomutase (EC 2.7.5.1) catalyzes the reversible transfer of a phosphate group between the 1 and 6 positions of glucose. Two different forms of the enzyme, the phospho form (E_P)[1] and the dephospho form (E_D) are involved in the transformation (Najjar and Pullman, 1954; Yankeelov *et al.*, 1964), which may be classified as a modified Ping-Pong reaction (Cleland, 1963a). Isotopic tracer studies carried out under initial velocity conditions indicate that the pathway must be formulated in such a manner that *free* glucose 1,6-bisphosphate is not an obligatory intermediate (Ray and Roscelli, 1964a), *i.e.*

$$\begin{array}{ccccc} E_P & & \left[\begin{array}{l} E_P\cdot\text{glucose-1-P} \\ E_D\cdot\text{glucose-1,6-P}_2 \\ E_P\cdot\text{glucose-6-P} \end{array}\right] & & E_P \\ + & \rightleftharpoons & & \rightleftharpoons & + \\ \text{glucose-1-P} & & & & \text{glucose-6-P} \end{array}$$

$$E_D + \text{glucose-1,6-P}_2 \qquad (1)$$

Since the phosphate group in E_P is esterified to the hydroxyl group of a serine residue (Kennedy and Koshland, 1957) the present investigation was initiated in order to evaluate the standard free-energy change accompanying hydrolysis of this group, $\Delta F°_{hyd}$, and to compare this value with corresponding values for other phosphoenzyme systems. Because of the unfavorable equilibrium (see Discussion), a direct measurement of $\Delta F°_{hyd}$ was not feasible; accordingly, phosphate transfer to two different acceptors (glucose-6-P and glucose-6-S) was measured directly and the results used to calculate $\Delta F°_{hyd}$. Three different and independent methods that are generally applicable in evaluating equilibria for Ping-Pong-type reactions were employed in measuring

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† Public Health Service Predoctoral Trainee.

‡ Career Development Awardee.

[1] Abbreviations used: PGM, phosphoglucomutase; E_P, phosphoenzyme; E_D, dephosphoenzyme; glucose-1-P, α-D-glucose 1-phosphate; glucose-6-P, D-glucose 6-phosphate; glucose-1,6-P_2, α-D-glucose 1,6-bisphosphate; glucose-6-S, D-glucose 6-sulfate; glucose-1-P-6-S, D-glucose 1-phosphate 6-sulfate; TCA, trichloroacetic acid; K_A, the Michaelis constant of substrate A or that concentration of A producing half-maximal velocity in the presence of saturating concentrations of all other substrates and in the absence of inhibitors; $K_{I(A)}$, the inhibition constant of A or the dissociation constant describing dead-end inhibition of the enzyme by A; $K_{x(A)}$, the isotope-exchange constant of A, or the concentration of A producing half-maximal isotope-exchange rate at saturating concentrations of all other reaction components; $K_{ix(Mg)}$ is an isotope-exchange constant used only for magnesium ion. It is analogous to the K_{ia}-type constants defined by Cleland (1963a) for initial velocity equations and is identical with the dissociation constant of the $E_P\cdot Mg$ complex, $K^1_{ix(Mg)}$, or of the $E_D\cdot Mg$ complex $K^{1,6}_{ix(Mg)}$; K_e, the equilibrium constant for glucose-1-P $\rightleftharpoons$ glucose-6-P; K_n, the equilibrium constant for eq *n*; r_0 and R, initial and maximum rates, respectively, for isotope exchange at equilibrium. Superscripts "*f*" and "*r*" are sometimes used to identify constants which have different values associated with the forward and reverse reactions, respectively, *e.g.*, R^f and $K^f_{x(\text{glucose-1,6-P}_2)}$. "app" is used to designate constants measured under specific conditions that are in turn functions of other constants and/or concentration terms, *e.g.*, $K^{f(app)}_{x(\text{glucose-1,6-P}_2)}$ is the concentration of glucose-1,6-P_2 producing half-maximal isotope-exchange rate in the forward reaction (glucose-1-P to glucose-6-P) at specified concentrations of glucose-1-P and Mg^{2+}.

phosphate transfer; two of these methods do not appear to have been previously used.

Experimental Section

Materials. Procedures for the preparation of PGM, glucose-1-P, glucose-6-P, and glucose-1,6-P_2 were described previously (Ray and Koshland, 1962; Ray and Roscelli, 1964a,b). A molecular weight of 62,000 (Filmer and Koshland, 1963) and an extinction coefficient of 7.7 for a 1% solution (Najjar, 1955) were used in all calculations. Muscle phosphorylase was purchased from Nutritional Biochemical Co. and dialyzed according to Cori *et al.* (1955) to remove contaminating P_i.

Glucose 6-sulfate was prepared according to Whistler *et al.* (1963) and purified by ion-exchange chromatography on a 1.3 × 17 cm column of Dowex 1 (see below). A linear gradient of pyridinium formate, pH 3.0, 0–2.25 M in 400 ml was used and the fractions containing glucose-6-S were located *via* the anthrone analysis (Barlett, 1959) and pooled; 1 equiv of KOH was added, and the material evaporated to dryness *in vacuo*. The product was crystallized twice according to Whistler *et al.* (1963) and stored over silica gel at −20°.

^{32}P-Labeled glucose-1-P was prepared from glycogen and [^{32}P]orthophosphate *via* the muscle phosphorylase reaction (Cori *et al.*, 1955). Alternatively an equilibrium mixture of ^{32}P-labeled glucose-1-P and glucose-6-P was prepared by including in the reaction a catalytic amount of PGM. The product or product mixture was purified *via* the ion-exchange chromatography and isolation procedures of Ray and Roscelli (1964a). ^{32}P-Labeled PGM was prepared (Ray and Koshland, 1963) and stored (Yankeelov *et al.*, 1964) as described previously.

Chromatography of Substrates and Products. All chromatography of glucose phosphates and glucose sulfates was performed on Dowex 1 (formate) resin, 8% cross-link, 200–400 mesh unless otherwise stated. The columns were eluted with a linear gradient of pyridinium formate (pH 3.0). Column dimensions and gradient characteristics are described in the appropriate section.

Standard Assay Procedures. Phosphoglucomutase activity was measured according to Ray *et al.* (1966) by means of the decrease in acid labile phosphate (decrease in glucose-1-P) produced by the enzymatic reaction. A pretreatment step (Ray and Roscelli, 1966a) was used in conjunction with the assay and all other enzyme reactions.

Organic [^{32}P]phosphate was assayed in the presence of $^{32}P_i$ by a modification of the precipitation method of Sugino and Miyoshi (1964). To a sample aliquot was added 0.1 μmole each of carrier P_i, glucose-1-P, and glucose-6-P, 6.9 μmoles of ammonium molybdate, and 8.5 μmoles of triethylamine hydrochloride; perchloric acid was added to a final volume of 1.35 ml and a molarity of 0.57. After heating for 10 min in a boiling-water bath to hydrolyze acid-labile phosphate (optional step, see below) the samples were cooled to 0° for at least 8 hr if >99.5% precipitation of P_i was required; however, samples could be used within 10 min when precipitation of 98–99% of the P_i was acceptable. The samples were filtered through cellulose acetate membranes (0.6-μ pore size, Schleicher & Schuell Co.) and aliquots of the filtrate containing the organic phosphate were plated on ringed aluminum planchets and dried under a heat lamp. To measure $^{32}P_i$ directly, the filter disk was washed with precipitating reagent (Sugino and Miyoshi, 1964) and glued to a flat planchet. Analysis with and without the optional hydrolysis step allowed distinction between acid-labile phosphate (glucose-1-P) and acid-stable phosphate (glucose-6-P). Samples were counted to within a 1% standard error with a Nuclear-Chicago low-background Geiger counter.

Direct Equilibrium Measurements Employing Glucose 6-Sulfate. A. REACTION WITH ^{32}P-LABELED PHOSPHOGLUCOMUTASE. ^{32}P-Labeled PGM (0.05–6.0 μg in 0.1 ml) was added to 0.8 ml of 80 mM histidine–25 mM Tris (pH 7.4 or 8.5), 1 mM in magnesium chloride. Chloride was used as the counterion in all of the buffer systems. After 10 min at 30° 0.1 ml of glucose-6-S (0.001–1.0 μmole) was added. Time aliquots, (0.1 ml) were removed and quickly mixed with 0.4 ml of carrier solution containing bovine serum albumin (1 mg/ml) and sodium glycerophosphate (1 mM), and 0.1 ml of 30% TCA was added. After standing on ice for 2 hr the samples were centrifuged and the supernatant was filtered through a cellulose acetate membrane (0.6-μ pore size, Schleicher & Schuell Co.). The filtrate was analyzed for total radioactivity (TCA soluble, counts per minute), $^{32}P_i$, and acid-labile [^{32}P]phosphate (see above).

B. EQUILIBRIUM BETWEEN GLUCOSE 1,6-BISPHOSPHATE AND GLUCOSE 1-PHOSPHATE 6-SULFATE. A substrate mixture (0.3 ml) containing either 33 μM glucose-6-S, 33 μM glucose-6-P, and 33 μM glucose-1,6-P_2, or a mixture with a 100-fold-increased concentration of glucose-6-S and glucose-6-P was added to 0.5 ml of 80 mM histidine–25 mM Tris (pH 7.4 or 8.5) which contained 1 mM magnesium chloride and catalytic amounts of PGM (10^{-7}–10^{-9} M). Approximately 5 μc of [^{32}P]glucose-1-P (or an equilibrium mixture of [^{32}P]glucose-1-P and [^{32}P]glucose-6-P) (specific activity, 1–20 mc/μmole) was added in 0.1 ml. After appropriate time intervals at 30° the reaction was stopped with 0.2 ml of 30% TCA; the mixture was diluted 25-fold with water containing 0.5 μmole each of glucose 1- and 6-phosphates and glucose-1,6-P_2 and chromatographed on a 0.6 × 15 cm column of Dowex 1 by using a formate gradient, 0–3.0 M in 500 ml. Peaks corresponding to the glucose monophosphates, glucose-1,6-P_2, and glucose-1-P-6-S were pooled and assayed for total radioactivity.

Equilibrium Isotope-Exchange Studies. The enzyme was preactivated (Ray and Roscelli, 1966a) at either pH 7.4 or 8.5 for 10 min at 30° in 0.3 ml of solution containing histidine (53 mM), Tris (17 mM), $MgCl_2$ (14.3–500 μM), and gelatin (0.01 mg/ml). Subsequently 0.15 ml of an equilibrium mixture containing glucose-1-P ($0.21–5 \times 10^{-3}$ μmole), glucose-6-P, and glucose-1,6-P_2 ($0.01–5 \times 10^{-3}$ μmole) was added and allowed to equilibrate for 15 min. Reactions were initiated by adding a constant but trace amount of [^{32}P]glucose-1-P, much less than 10^{-4} μmole, in 0.05 ml. After 10 min reactions were stopped with 0.5 ml of 4% $HClO_4$. The acid-stable

[^{32}P]phosphate produced was determined by the precipitation method (see above). Enzyme concentrations at each point were adjusted to produce a constant amount of acid-stable [^{32}P]phosphate, 10 ± 3% of the total radioactivity. The blank (no enzyme) contained 0.05% or less of the total radioactivity.

Phosphate Binding by Dephosphoenzyme. INHIBITION STUDIES. PGM was preactivated (Ray and Roscelli, 1966a) in 0.9 ml of 80 mM histidine–25 mM Tris (pH 8.5), 0.15 mM in magnesium chloride. Reactions were started by adding 0.1 ml of a substrate mixture containing glucose-1-P (2.5×10^{-3} M), [^{32}P]glucose-1-P (1 μc; specific activity, 20c/mmole), glucose-1,6-P_2 (2.5×10^{-7}–5×10^{-4} M), and orthophosphate (0–30 mM). PGM concentrations were varied to produce the same percent conversion of substrate to product (30%) in a given time (20 min). Since the PGM system produces a burst of product prior to the steady-state phase when the reaction is carried out at subsaturating Mg^{2+} and glucose-1,6-P_2 (Ray and Roscelli, 1966b), velocities in this study were measured in terms of the product (acid-stable phosphate) produced between 2 and 22 min, *i.e.*, in the linear phase of the reaction.

CALCULATION OF KINETIC CONSTANTS. Kinetic constants and their standard deviations were calculated with an IBM 7090 computer by using a modified version (Ray and Roscelli, 1966a) of a program (Cleland, 1963b) for fitting data by the method of least squares to an equation analogous to eq 16.

Theory

Two different equilibria are involved in Ping-Pong-type reactions.

$$E + A \rightleftharpoons F + P \quad (2)$$

$$F + B \rightleftharpoons E + Q \quad (3)$$

E and F are different forms of the enzyme while substrates are A and B and products are P and Q (Cleland, 1963a). Each of the following three procedures can be used to evaluate the constants for both of these equilibria.

Direct Measurement. If the equilibrium concentrations of A, P, E, and F can be evaluated, the equilibrium constant for eq 2 may be determined directly provided that either (a) the concentration range used is sufficiently low so that enzyme–substrate complexes, *e.g.*, E·A, are unimportant, or (b) the analytical procedures used can distinguish between the free and complexed forms of the reactants, *e.g.*, between E and E·A. Since alternative b is not feasible in the phosphoglucomutase system, only the former possibility is considered in the following discussion.

To determine whether enzyme–substrate complexes are present under conditions used for equilibrium measurements, various limiting cases may be tested by examining equilibrium constants based on the conditions to produce that case. Thus the limiting cases for eq 4

$$E + A \underset{}{\overset{K_4}{\rightleftharpoons}} E\cdot A \overset{K_4'}{\rightleftharpoons} F\cdot P \overset{K_4''}{\rightleftharpoons} F + P \quad (4)$$

are given below, where $[E_T]$ and $[A_0]$ are the known concentrations of reactants and where [P] is the measured value of $[F\cdot P] + [P]$, *i.e.*, in an analytical procedure that does not distinguish between the two.

(a) No enzyme–substrate complexes are present.

$$K_4 \gg [A_0];\ K_4'' \gg [E_T]$$

$$K_{app} = K_4'K_4''/K_4 = [P]^2/([A_0] - [P])([E_T] - [P]) \quad (5)$$

(b) No free E is present.

$$K_4 \ll [A_0];\ K_4'' \gg [E_T]$$

$$K_{app} = K_4'K_4'' = [P]^2/([E_T] - [P]) \quad (6)$$

(c) No free F is present.

$$K_4 \gg [A_0];\ K_4'' \ll [E_T]$$

$$K_{app} = K_4'/K_4 = [P]/([E_T] - [P])([A_0] - [P]) \quad (7)$$

(d) No free E or F is present.

$$K_4 \ll [A_0];\ K_4'' \ll [E_T]$$

$$K_{app} = K_4' = [P]/([E_T] - [P]) \quad (8)$$

An invariance in the right-hand side of eq 5, 6, 7, or 8 when both $[A_0]$ and $[E_T]$ are varied by tenfold in such a way that the $[A_0]/[E_T]$ ratio is altered by 100-fold is sufficient to verify the limiting condition under which that equation was derived. Obviously the desired condition, case a, is the limiting case obtained as both enzyme and substrate concentrations approach zero.

The advantage of a direct measurement is its relative rapidity; disadvantages are the requirement of a pure sample of one form of the enzyme to the exclusion of the other plus a knowledge of the molar concentration of the enzyme. In addition, if more than one catalytic site is present the system becomes much more complex; also, if either the substrate or product are tightly bound, working at sufficiently low concentrations[2] so that enzyme–substrate complexes are unimportant may impose technical problems. In the phosphoglucomutase system the latter requirement precluded the use of the normal substrate, glucose-6-P; a synthetic substrate, glucose-6-S, which is much less firmly bound by the enzyme was used instead.

Equilibrium Isotope-Exchange Studies. In Ping-Pong

[2] In general, neither enzyme nor reactant should be used at a concentration greater than 0.05 of the smaller enzyme–substrate dissociation constant.

reactions the conversion of A to P can be followed at equilibrium in the absence of B and Q by use of a radioactive label (Boyer, 1959). The initial isotopic exchange rate (r_0) in such a case is given by

$$r_0 = R_{max}\left[1 + \frac{K_{x(A)}}{[A]} + \frac{K_{x(P)}}{[P]}\right]^{-1} \quad (9)$$

when [A] and [P] $\gg$ [E_T]. Here R_{max} is the maximum isotopic exchange rate at equilibrium and the K_x's are the concentrations of A and P giving the half-maximum exchange rate in the presence of saturating concentrations of the other.[3] Since $K_{x(A)}$ = [E][A]/[CC] and $K_{x(P)}$ = [F][P]/[CC], the ratio $K_{x(P)}/K_{x(A)}$ is the equilibrium constant for eq 2. In these expressions [CC] represents the central complex, *i.e.*, the sum of [E·A] and [F·P] in eq 4. Equations similar to these are derived by Boyer (1959) for somewhat different cases. The values of $K_{x(A)}$ and $K_{x(P)}$ can be determined by the use of double-reciprocal plots as described by Ray *et al.* (1966).

In the case of phosphoglucomutase the first product, glucose-1,6-P_2, is identical with the second reactant (Ray and Roscelli, 1964a), *viz.*, in terms of eq 2 and 3 P ≡ B; hence the two steps in the enzymic reaction cannot be investigated separately. However, in such a case, although the derivation is slightly different and the reaction in question, A* → Q*, is also different, $K_{x(P)}/K_{x(A)}$ is still equal to K_2, *viz.*, $K^f_{x\ \text{glucose-1,6-P}_2}/K_{x(\text{glucose-6-P})}$ = [E_D][glucose-1,6-P_2]/[E_P][glucose-6-P]. The advantage of this type of procedure lies in the possibility of using substrates at concentrations much higher than that of the enzyme. This eliminates all of the disadvantages noted for the previous method; however, the actual collection of data may be technically more difficult.

Initial Velocity Studies. According to Cleland's nomenclature, $K_{i(P)}$ and $K_{i(A)}$ are, respectively, the inhibition constant for P in the forward reaction (A + B → P + Q) and the inhibition constant for A in the reverse reaction. It can be shown (see Appendix) that

$$K_{i(P)}/K_{i(A)} = [F][P]/[E][A] \quad (10)$$

regardless of the number of central complexes involved in the reaction; hence, the equilibrium constant in question can be determined from product inhibition studies. An analogous equation can be derived for [E][Q]/[F][B] (see Appendix).

However, for the phosphoglucomutase reaction there are no $K_{i(P)}$ and $K_{i(B)}$ terms because of the first product–second substrate identity noted above. A derivation similar to that in the Appendix gives the following expression.

[3] $K_{x(A)}$ and $K_{x(P)}$ are identical with the K_{ia} and K_{ip} constants that appear in initial velocity equations for *normal* Ping-Pong reactions as formulated by Cleland (1963a); see also Cleland (1967); however, in the phosphoglucomutase system there are no K_{ia}- or K_{ip}-type constants (see below).

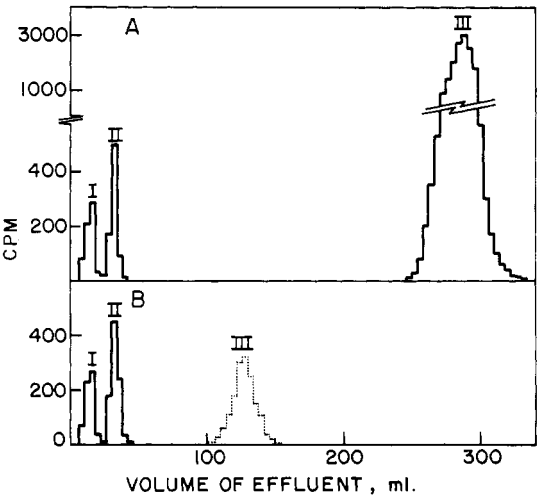

FIGURE 1: Ion-exchange chromatography (see Experimental Section) of the products from the reaction of [^{32}P]PGM with glucose-6-S. Gradient, 0.5–3.0 M in 500 ml. Part A is the elution pattern from a deproteinized sample of a reaction mixture containing [^{32}P]PGM and glucose-6-S which had been incubated for 60 min at 30° in an 80 mM histidine–25 mM Tris buffer (pH 7.4). Part B is a control treated identically but without glucose-6-S. The dotted line in B indicates the position at which glucose-1,6-P_2 appears in this chromatographic system.

$$\frac{[E_D][\text{glucose-1,6-P}_2]}{[E_P][\text{glucose-6-P}]} = \frac{K^f_{\text{glucose-1,6-P}_2}}{K_e K_{\text{glucose-1-P}}} + \frac{K^r_{\text{glucose-1,6-P}_2}}{K_{\text{glucose-6-P}}} \quad (11)$$

The superscripts *f* and *r* refer, respectively, to parameters measured in the forward and reverse reactions, and K_e is [glucose-6-P]/[glucose-1-P]; hence the required equilibrium constant K_{15} (see below) can be evaluated from initial velocity studies in the forward and reverse reactions. The advantages and disadvantages of this method are similar to those noted above for the equilibrium isotope-exchange procedure.

Results

Equilibrium Constant for Phosphate Transfer from Phosphoenzyme to Glucose-6-P. EVALUATION *via* DIRECT MEASUREMENT WITH GLUCOSE 6-SULFATE. When ^{32}P-labeled PGM is treated with massive amounts of glucose 6-sulfate, all of the [^{32}P]phosphate originally bound covalently to the enzyme (*i.e.*, TCA-precipitable radioactivity) is found in the TCA-soluble reaction products. Figure 1 compares column chromatograms of the TCA-soluble label (A) with that from a control lacking glucose-6-S (B). All of the ^{32}P-label in the main peak (IIIA) is acid labile; hence, the label is attached to the 1 position of the glucose ring. Moreover, the material in peak IIIA is more firmly bound to the anionic resin at pH 3

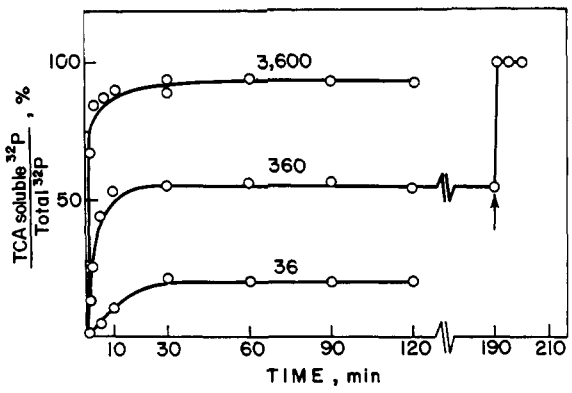

FIGURE 2: Plot of per cent ^{32}P-label released from [^{32}P]PGM as a function of time at three concentrations of glucose-6-S. The reaction was carried out with 1.0 mμM enzyme under conditions described in the Experimental Section. The numbers on the lines refer to the ratio, [glucose-6-S]/[E_T]. The arrow indicates an addition of excess glucose-6-S (1 mmole). Each point represents triplicate assays; standard error $\pm 3\%$.

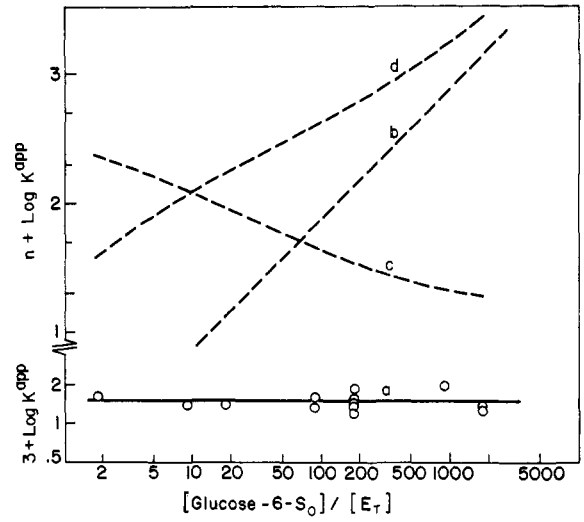

FIGURE 3: Plots of log K^{app} *vs.* a logarithmic function of the glucose-6-S:enzyme ratio as a test for the presence of enzyme–substrate complexes. Lines a–d show K^{app} calculated according to assumptions a–d (see Theory section). All plots involve the same ordinate scale; however, because of the range of K^{app} values involved, lines b–d have been transposed by appropriate adjustment of n. Data for calculation of K^{app} were obtained from experiments similar to those in Figure 2. Each point represents duplicate or triplicate assays; standard error $\pm 3\%$.

than is glucose-1,6-P_2 (see Figure 1B). Hence we conclude that peak IIIA is the mixed diester, glucose 1-phosphate 6-sulfate, and that the reaction in question is

$$[^{32}P]E_P + \text{glucose-6-S} \rightleftharpoons [^{32}P]\text{glucose-1-P-6-S} + E_D \quad (12)$$

The relatively small peaks (I and II) in the early portion of both chromatograms are glucose monophosphate and P_i, respectively. Both were present in the original [^{32}P]PGM sample (5% of the total label) and were unchanged by the glucose-6-S reaction. This shows that a further reaction of the product with E_D, *viz.*

$$E_D + [^{32}P]\text{glucose-1-P-6-S} \rightleftharpoons [^{32}P]\text{glucose-1-P} + \text{sulfoenzyme} \quad (13)$$

does not occur under these conditions.

Figure 2 illustrates the rate of approach to equilibrium for the reaction described in eq 12 when limiting amounts of glucose-6-S were incubated with [^{32}P]PGM. In one case, after the attainment of equilibrium at an intermediate concentration of glucose-6-S, a massive amount of glucose-6-S was added to demonstrate the viability of the enzyme system. As expected, this addition caused the release from the enzyme of all of the remaining ^{32}P label.

Tests for the presence of enzyme–substrate complexes[4] in the reaction mixture, carried out as outlined in the Theory section, demonstrated that neither glucose-6-S nor glucose-1-P-6-S is appreciably bound by the enzyme under the conditions employed. This is shown in Figure 3 which gives plots of log K^{app} (for definition see Theory section) *vs.* a logarithmic function of the substrate:enzyme ratio. Values of K^{app} calculated on the basis of assumptions b, c, and d vary with the concentration of reactants as shown by the dashed lines; conversely, values calculated on the basis of a are

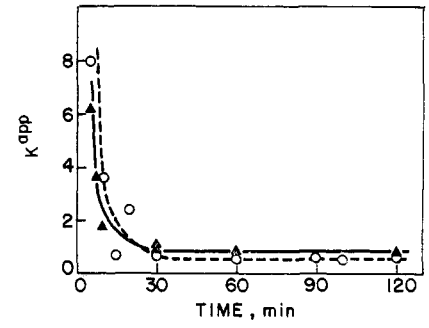

FIGURE 4: Plot demonstrating approach to equilibrium for the reaction: glucose-1,6-P_2 + glucose-6-S $\rightleftharpoons$ glucose-1-P-6-S + glucose-6-P catalyzed by PGM. For details of the reaction, see the Experimental Section. K^{app} was calculated *via* eq 5. The abscissa (time) is normalized for varying enzyme concentrations; (----) data obtained at pH 8.5; (——) data obtained at pH 7.4. Each point represents a single assay.

[4] Glucose-6-S is considered a substrate for E_P while glucose-1-P-6-S is a substrate for E_D.

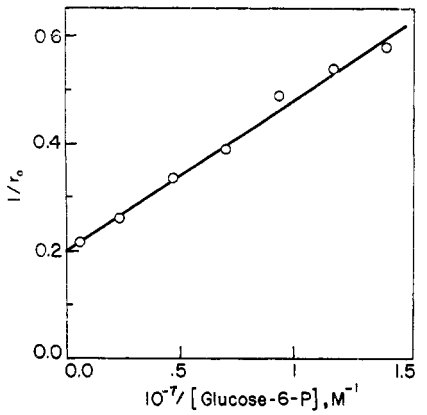

FIGURE 5: Double-reciprocal plot of isotope-exchange rate (r_0) and glucose-6-P concentration at saturating glucose-1,6-P_2 (0.01 mM) and saturating Mg^{2+} (0.3 mM). For conditions see the Experimental Section. Kinetic constants are $K_{x(\text{glucose-6-P})} = 14 \pm 0.9$ μM, $R^f = 0.0497 \pm 0.0014$ μmole/μg min. The solid line was drawn by using these values. Each point represents a single assay.

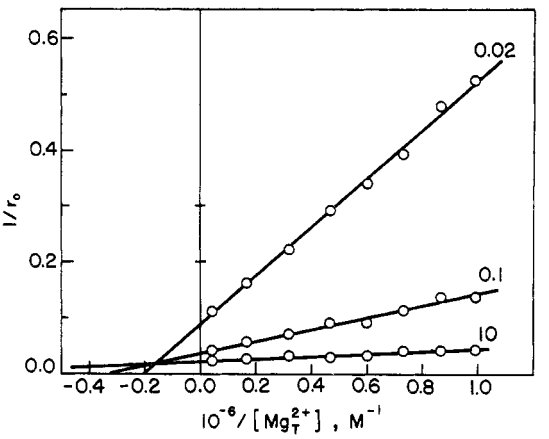

FIGURE 6: Double-reciprocal plots of r_0 and Mg^{2+} concentration for three subsaturating glucose-1,6-P_2 concentrations (numbers on plots represent concentrations of glucose-1,6-P_2 in micromolar at saturating glucose-1-P (0.03 mM) (pH 8.5)). For conditions, see the Experimental Section. Kinetic constants are $K^{app}_{x(\text{glucose-1,6-P}_2)} = 0.074 \pm 0.011$ μM, $K^{1,6}_{ix(\text{Mg})} = 0.057 \pm 0.007$ mM, and $R^f = 0.0523$ μmole/μg min. The solid lines were drawn by using these values. Each point represents a single assay.

constant while substrate concentration is varied 100-fold, enzyme concentration is varied tenfold and the substrate:enzyme ratio is varied 1000-fold. The average value of K^{app}, *viz.*, K_{12}, was $1.5 \pm 0.2 \times 10^{-3}$ at pH 8.5 and $0.27 \pm 0.03 \times 10^{-3}$ at pH 7.4 (latter data not shown). In these experiments the Mg^{2+} concentration (1 mM) was sufficient to saturate both E_P and E_D (Ray and Roscelli, 1966b). If we assume that glucose-6-S does not bind Mg^{2+} more tenaciously than does glucose-6-P[5] and that glucose-1-P-6-S does not bind Mg^{2+} more tenaciously than the trianion of glucose-1,6-P_2 (Ray and Roscelli, 1966a) the concentration of neither of the sulfate-containing substrates is appreciably affected by the concentration of Mg^{2+} used.

Figure 4 shows the approach to equilibrium in systems containing glucose-6-P, glucose-1,6-P_2, and glucose-6-S, *viz.*

$$\text{glucose-1-P-6-S} + \text{glucose-6-P} \rightleftharpoons \text{glucose-1,6-P}_2 + \text{glucose-6-S} \quad (14)$$

The equilibrium constant (K_{14}) is 0.67 ± 0.02 at pH 8.5 and 0.85 ± 0.03 at pH 7.4. The concentration of Mg^{2+} used here (0.1 mM) was sufficiently low so that essentially no Mg^{2+} complexes of glucose-1,6-P_2 (Ray and Roscelli, 1966a) were present. Since, of the species present, the bisphosphate probably binds Mg^{2+} most strongly (see above) the level of Mg^{2+} used here is too low to appreciably affect K_{14}.

The product of K_{14} and K_{12} is equal to K_{15}.[6]

$$E_P + \text{glucose-6-P} \rightleftharpoons E_D + \text{glucose-1,6-P}_2 \quad (15)$$

Hence $K_{15} = 1.0 \times 10^{-3}$ at pH 8.5 and 0.22×10^{-3} at pH 7.4.

EVALUATION WITH THE EQUILIBRIUM ISOTOPE-EXCHANGE PROCEDURE. For the PGM reaction pathway described by Ray *et al.* (1966), the isotopic exchange rate at equilibrium (Boyer, 1959) is given by

$$\frac{R^f}{r_0} = 1 + \frac{K_{x(\text{Mg})}}{[Mg^{2+}]} + \frac{K_{x(\text{glucose-6-P})}}{[\text{glucose-6-P}]}\left\{1 + \frac{K^1_{ix(\text{Mg})}}{[Mg^{2+}]}\right\} + \frac{K_{x(\text{glucose-1,6-P}_2)}}{[\text{glucose-1,6-P}_2]}\left\{1 + \frac{K^{1,6}_{ix(\text{Mg})}}{[Mg^{2+}]} + \frac{[\text{glucose-P}]}{K_{I(\text{glucose-P})}}\right\} \quad (16)$$

The system used here for designating constants is analogous to that used in defining the initial velocity equation for the PGM system (Ray *et al.*, 1966) except that constants in the above equation include an additional subscript, "x" to identify the constant as an isotope-exchange constant. Since both glucose-1-P and glucose-6-P are present at equilibrium, [glucose-P] refers to the sum of the concentrations of both, and $K_{I(\text{glucose-P})}$ is the dead-end inhibition constant[7] for an equilibrium mixture of the glucose monophosphates.

$K_{x(\text{glucose-6-P})}$ was measured at pH 8.5 in the manner described previously (Ray *et al.*, 1966) in the presence of sufficient Mg^{2+} and glucose-1,6-P_2 to saturate the en-

[5] Sulfate binds Mg^{2+} less tenaciously than phosphate (Clarke *et al.*, 1954).

[6] Note that previous investigators have reported their results in terms of $1/K_{15}$.

[7] Dead-end inhibition (Cleland, 1963a) competitive with glucose-1,6-P_2 is also produced by glucose-1-P and glucose-6-P under initial velocity conditions (see following section).

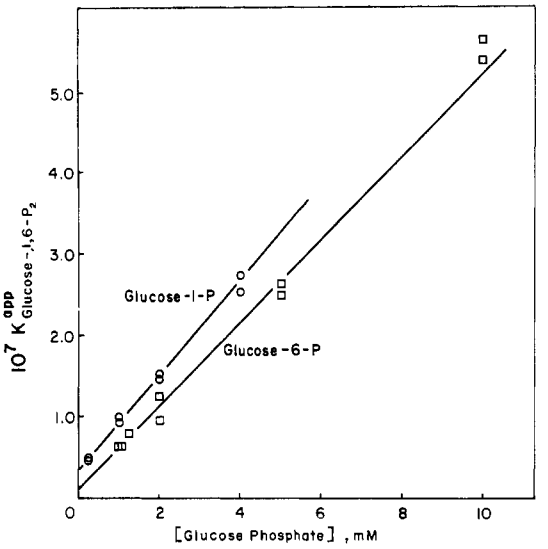

FIGURE 7: $K^{app}_{glucose\text{-}1,6\text{-}P_2}$ as a function of glucose-1-P (○) and glucose-6-P (□). The data for $K^{r\,(app)}_{glucose\text{-}1,6\text{-}P_2}$ when glucose-6-P was the substrate have been reported previously (Ray and Roscelli, 1964a); the data for $K^{f\,(app)}_{glucose\text{-}1,6\text{-}P_2}$ when glucose-1-P was the substrate were obtained and analyzed in an identical manner using the same assay conditions; however, in the latter case initial velocities were measured directly instead of using the approximations previously used in the evaluation of $K^{r\,(app)}_{glucose\text{-}1,6\text{-}P_2}$.

zyme. Figure 5 shows the appropriate double-reciprocal plot; the slope:intercept ratio, $K_{x(glucose\text{-}6\text{-}P)}$, is 14 μM.

A similar though more complex procedure was used to evaluate $K_{x(glucose\text{-}1,6\text{-}P_2)}$. Thus isotope-exchange rates were measured at 10–300 μM Mg^{2+} (subsaturating concentrations) in the presence of 0.51 mM glucose-6-P (a saturating concentration). The results are shown as double-reciprocal plots in Figure 6. Appropriate replots (Cleland, 1963a) are linear as expected from eq 15: $K^{app}_{x(glucose\text{-}1,6\text{-}P_2)} = 0.074 \pm 0.011$ μM (at saturating Mg^{2+}). Under these conditions

$$K^{app}_{x(glucose\text{-}1,6\text{-}P_2)} = K_{x(glucose\text{-}1,6\text{-}P_2)}\left\{1 + \frac{[glucose\text{-}P]}{K_{I(glucose\text{-}P)}}\right\} \quad (17)$$

Since $K_{I(glucose\text{-}P)}$ is about 1 mM,[8] $K_{x(glucose\text{-}1,6\text{-}P_2)}$ is 0.047 μM.

The ratio $K_{x(glucose\text{-}1,6\text{-}P_2)}/K_{x(glucose\text{-}6\text{-}P)}$ (see Theory section) is equal to K_{15} or 3.1×10^{-3}. Note that both K_x values refer to processes occurring at saturating Mg^{2+} (relative to the enzyme) since the relevant experiments were carried out so that essentially no Mg^{2+} complexes of the varied substrate were present.

EVALUATION WITH DATA FROM INITIAL VELOCITY STUDIES. The apparent Michaelis constants for glucose-1,6-P_2 in the forward and reverse reactions under identical assay conditions are shown in Figure 7 as a function of glucose-1-P and glucose-6-P, respectively. In both plots monophosphate concentrations were above saturation and the ratio of the y intercepts of the two plots thus gives the ratio of the two Michaelis constants corrected for dead-end substrate inhibition (Ray and Roscelli, 1964a; Ray *et al.*, 1966). However, the extrapolated value of $K^{f\,(app)}_{glucose\text{-}1,6\text{-}P_2}$ (3×10^{-8} M) is significantly larger than the value previously given for $K^{f}_{glucose\text{-}1,6\text{-}P_2}$ (1×10^{-8} M) (Ray *et al.*, 1966). This is because both Mg^{2+} and sulfate also inhibit competitively with respect to glucose-1,6-P_2 and both were present at inhibiting concentrations in all assays. However, since Mg^{2+} inhibits by reducing the concentration of free glucose-1,6-P_2 and sulfate inhibits by binding to E_D (Ray and Roscelli, 1966a), both should have the same relative effect on Michaelis constants for glucose bisphosphate in the forward and reverse reactions; hence, the ratio $K^{f}_{glucose\text{-}1,6\text{-}P_2}/K^{r}_{glucose\text{-}1,6\text{-}P_2}$ is equal to the y-intercept ratio in Figure 7, *viz.*, about 3, and $K^{f}_{glucose\text{-}1,6\text{-}P_2}$ is thus about 3×10^{-9} M.

By using $K_{glucose\text{-}1\text{-}P} = 5$ μM (Ray *et al.*, 1966), $K_{glucose\text{-}6\text{-}P}/K_{glucose\text{-}1\text{-}P} = 6$ (Ray and Roscelli, 1964b), $K_e = 17$ (Colowick and Sutherland, 1942), together with the above values for $K^{f}_{glucose\text{-}1,6\text{-}P_2}$ and $K^{r}_{glucose\text{-}1,6\text{-}P_2}$, the value of K_{15} at pH 7.4 is about 2×10^{-4} M (see eq 11). The effect of Mg^{2+} on this constant is the same as that outlined in the previous section. The accuracy of K_{15} determined in this manner is questionable in view of the errors inherent in an evaluation involving so many different constants, several of which represent extrapolated values; however, the value obtained certainly establishes the order of magnitude of the constant at this pH.

Binding of Orthophosphate by Dephosphoenzyme via Inhibition Studies. The inhibition of PGM by P_i was examined under conditions where the inhibition is strictly competitive with glucose-1,6-P_2. For such experiments glucose-1-P must be maintained at a concentration sufficient to saturate the phosphoenzyme ($K_{glucose\text{-}1\text{-}P} = 5 \times 10^{-6}$ M; Ray *et al.*, 1966) but at a concentration below that which would produce appreciable dead-end inhibition *via* binding to the dephosphoenzyme ($K_{I(glucose\text{-}1\text{-}P)} \approx 1$ mM). The concentration used (2.5×10^{-4} M) fulfills both requirements reasonably well.

Figure 8 shows a plot of $1/v$ *vs.* 1/[glucose-1,6-P_2] at varying levels of orthophosphate; the inset of Figure 8 shows a replot of slopes *vs.* concentration of orthophosphate from Figure 8. The value of $K_{I(P_i)}$ is thus about 1 mM. Since Mg^{2+} concentration is much below the dissociation constant of the $Mg \cdot P_i$ complex (Clarke *et al.*, 1954) but substantially above the dissociation constant for $E_D \cdot Mg$, $K_{I(P_i)}$ refers to binding of P_i by $E_D \cdot Mg$.

Discussion

Three groups of investigators have reported values of the equilibrium constant for transfer of phosphate from

[8] D. Kirkpatrick, Ph.D. thesis.

E_P to glucose-6-P (eq 15) at pH 7.4: 3.76[9] (Hashimoto and Handler, 1966), 0.265 (Sidbury and Najjar, 1957), and <0.025 (Gounaris *et al.*, 1967). It is not clear whether all of the differences among these values are the result of failure to recognize and deal with the problem of enzyme–substrate complexes; however, in each case such complexes were undoubtedly present at significant levels. By contrast the close agreement (see Table I)

TABLE I: Thermodynamic Parameters for Transfer of Phosphate from Phosphoglucomutase to Glucose 6-Phosphate.[a]

Method	pH	K_{15}[b]	ΔF°
Substrate analog	8.5	1.0×10^{-3}	4.2
Isotope exchange	8.5	3.1×10^{-3}	3.5
Substrate analog	7.4	2.2×10^{-4}	5.1
Initial velocity	7.4	2.0×10^{-4}	5.1

[a] Comparison of values from three different methods. [b] $K_{15} = [E_D][\text{glucose-1,6-P}_2]/[E_P][\text{glucose-6-P}]$.

between values of K_{15} obtained from three independent approaches, each of which was designed specifically to circumvent this problem, suggests that the present results approximate the true value of the constant in question (see Table I). Note that the results are independent of the number or type of enzyme–substrate complexes involved in the phosphoglucomutase reaction.

A measured equilibrium constant will be a function of Mg^{2+} concentration in a system in which there is a significant difference in the binding of Mg^{2+} by two or more moieties participating in the equilibrium. All data for the PGM system have been collected and treated in such a way that the pertinent equilibria involve the Mg^{2+} complexes of both forms of the enzyme, and the free forms of all substrates. However, to simplify the presentation, Mg^{2+} will not appear in the following equations.

The sum of the standard free-energy changes for eq 15 and 18

$$\text{glucose-1,6-P}_2 + H_2O \rightleftharpoons P_i + \text{glucose-6-P} \quad (18)$$

is equal to the free-energy change accompanying hydrolysis (ΔF°_{hyd}) for

$$E_P + H_2O \rightleftharpoons E_D + P_i \quad (19)$$

However, the ΔF° value for eq 18 would be difficult to measure directly and the presence of charge effects precludes the assumption that ΔF°_{hyd} for the 1-phosphate in glucose-1,6-P_2 is the same as that for glucose-1-P.

[9] Although Hashimoto and Handler (1966) report a value of 3.76 for $1/K_{15}$, it can be shown from their data that the constant they report is actually K_{15}.

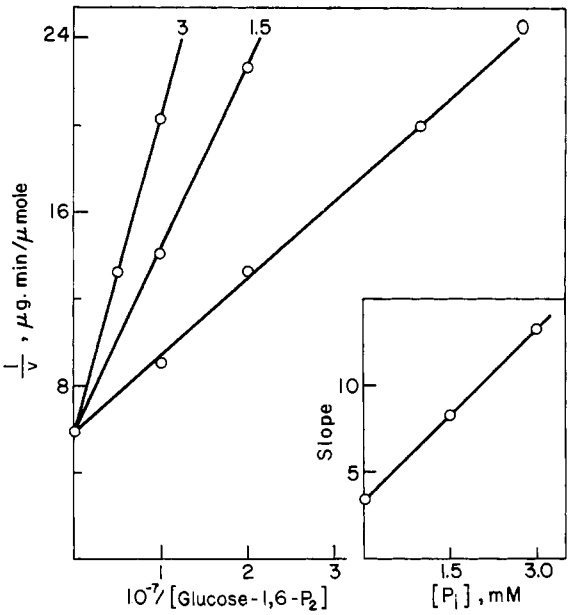

FIGURE 8: Double-reciprocal plots of velocity and glucose-1,6-P_2 at saturating glucose-1-P (0.25 mM) and at three concentrations of P_i (numbers on plots represent millimolar concentrations of P_i). Inset, plot of slopes from Figure 8 *vs.* P_i concentration; $K_{I(P_i)}$ = 1.0 mM. Each point represents a single assay.

Thus the presence of the dianionic 6-phosphate group in the bisphosphate should decrease the ΔF°_{hyd} for its 1-phosphate group relative to that of glucose-1-P. This is demonstrated by the preference of the phosphate group for attachment to the 1 position of glucose-6-S (monoanionic group in the 6 position) as opposed to the corresponding position of glucose-6-P (dianionic group in the 6 position). The measured free-energy difference between these two positions of attachment, 0.3 kcal, is the value predicted from solution theory. Thus the energy decrease in moving two dinegative groups from positions 9 Å apart to positions where their mutual repulsion is negligible can be approximated (Fuoss, 1958) under the conditions employed (ionic strength = 0.045, T = 30°).[10] The calculation involves many approximations (Hammes and Steinfeld, 1962) and the extent of agreement with the observed results is undoubtedly fortuitous. However such calculations indicate that ΔF°_{hyd} for the 1-phosphate in glucose-1,6-P_2 should be more negative than that for glucose-1-P by about twice the measured difference between glucose-1,6-P_2 and glucose-1-P-6-S, *viz.*, 0.6 kcal.[11]

[10] The value of 9 Å is the maximum separation attainable for phosphate or sulfate groups attached to the 1 and 6 positions of glucose as estimated from molecular models.

[11] Meyerhof and Green (1949) have reported an analogous difference for the 6 position of fructose mono- and bisphosphates, *i.e.*, $\Delta F^\circ_{hyd} = -3.4$ and -3.7 kcal for mono- and bisphosphates, respectively; a 0.3-kcal difference is thus due to the presence of the dianionic phosphate group in the bisphosphate.

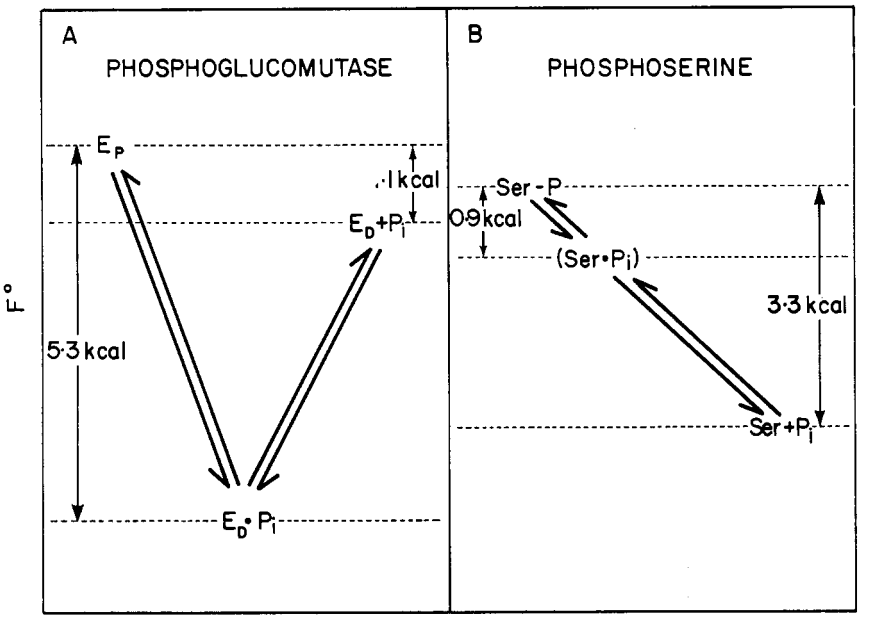

FIGURE 9: Free-energy diagram for the hydrolysis and dissociation of the phosphate group of PGM according to eq 20 and 21 (A) and for the hypothetical two-step hydrolysis of phosphoserine (B).

Of the two reported values for ΔF°_{hyd} of glucose-1-P, −4.2 kcal by Ginodman (1954) and −4.9 kcal by Meyerhof and Green (1949), the value of Ginodman is used here because his value was obtained in much more dilute solutions. By using the above data, values of ΔF°_{hyd} for the 1-phosphate of glucose-1,6-P_2 and glucose-1-P-6-S become −4.8 and −4.5 kcal, respectively. Since ΔF° for eq 15 is +3.7 kcal at pH 8.5 (value derived from average of K_{15} values in Table I) where ionization of the phosphate group should be complete, ΔF°_{hyd} for E_P is about −1.1 kcal; however, the error in this value may be as large as ±0.5 kcal.

Values of ΔF°_{hyd} are not ideal parameters for either comparing phosphate ester bonds in different phosphoproteins or making comparisons between phosphoproteins and simple phosphate esters, *e.g.*, serine phosphate. Thus ΔF°_{hyd} for the phosphate group of phosphoserine is −3.3 kcal (Vladimirova *et al.*, 1961)[12] while the corresponding values for the phosphate hydrolysis of phosvitin, phosphoglucomutase, and alkaline phosphatase are *ca.* −6, −1.1, and +5.5 kcal, respectively, in spite of the fact that the latter three are all phosphoserine proteins (Rabinowitz and Lipmann, 1960; Milstein and Sanger, 1961; Schwartz *et al.*, 1963). These differences may arise from elimination of noncovalent interactions between the protein and the covalently attached phosphate group during or subsequent to bond cleavage. Such interactions might be negative, as in the case of phosvitin (negative interactions could be rationalized in terms of charge–charge repulsions) or positive, as in the case of alkaline phosphatase (see below). Hence, to the extent that such interactions exist in phosphoproteins, ΔF°_{hyd} will be an unreliable basis for making comparisons, especially comparisons designed to deduce the nature of the phosphate–protein bond.

A somewhat better though still not ideal basis for comparing phosphate bonds can be obtained by separating the over-all hydrolysis into two steps, bond cleavage and dissociation of products, eq 20 and 21, respectively.

$$E_P + H_2O \rightleftharpoons E_D \cdot P_i \qquad (20)$$

$$E_D \cdot P_i \rightleftharpoons E_D + P_i \qquad (21)$$

Here $E_D \cdot P_i$ is the complex of the dephosphoprotein and orthophosphate. Experimentally, ΔF° for bond cleavage, eq 20, is the difference between ΔF°_{hyd} and ΔF° for separation of products, *i.e.*, $-\Delta F^{\circ}$ for phosphate binding. In the case of phosphoglucomutase ΔF°_{hyd} is −1.1 kcal and ΔF° for binding of phosphate is −4.2 kcal (see results); hence ΔF° for bond breaking is −5.3 kcal. Figure 9A is an energy-level diagram showing these steps; the free energy of $E_D + P_i$ in this diagram has arbitrarily been taken as zero.

Such a division is not straightforward for simple phosphate esters such as serine phosphate since the equilibrium $Ser \cdot P \rightleftharpoons Ser + P_i$ cannot be easily measured.

[12] Vladimirova *et al.* (1961) give equilibrium constants of 673 and 287 for hydrolysis of serine phosphate at pH 7 and 9.7, respectively. By using the following pK_a values (see Sillen and Martell, 1964) the equilibrium constant for $^{2-}O_3POCH_2CHNH_3^+$-$CO_2^- \rightleftharpoons HPO_4^{2-} + HOCH_2CHNH_3^+CO_2^-$ can be calculated as 430 and 200 from the data at pH 7.0 and 9.7, respectively; pK_2 of orthophosphate = 6.8; pK_3 of serine phosphate = 5.7; pK_2 of serine = 9.2; pK_4 of serine phosphate = 9.7. If an average of these two equilibrium constants is used, ΔF°_{hyd} for the above reaction is −3.3 kcal.

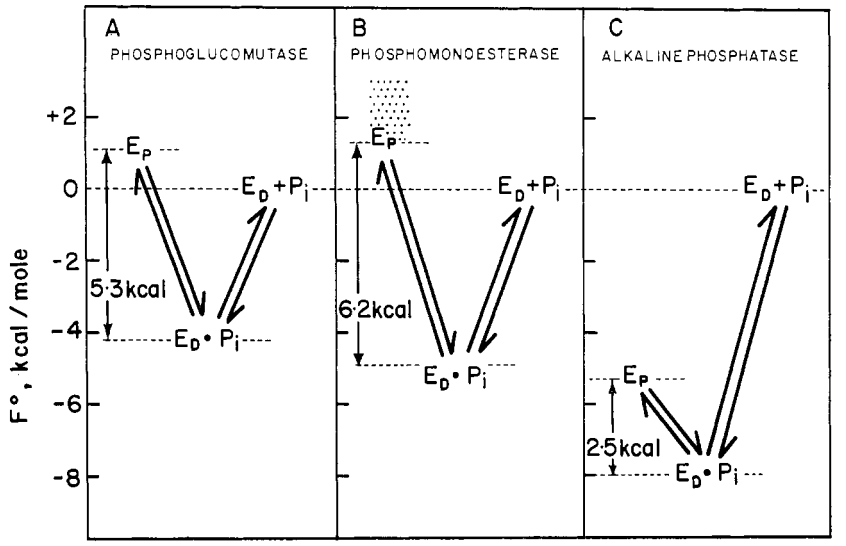

FIGURE 10: Comparison of free-energy diagrams for the hydrolysis and dissociation of the phosphate group of three phosphoproteins: PGM, phosphomonoesterase (data of Hsu *et al.*, 1966) and alkaline phosphatase (data of Wilson and Dayan, 1965).

However, if no alteration of charge occurs in either Ser or P_i moieties during formation or dissociation of the Ser·P_i complex, an estimate of this dissociation constant (55 M) can be made (Lumry, 1959; Epand and Wilson, 1964), provided that the activity of serine in the formation of the Ser·P_i complex in dilute solution is equal to its molarity. (This assumes that water competes equally with the hydroxyl group of serine on a mole-to-mole basis in complexing with or solvating P_i.) In such a case $\Delta F°$ for Ser·P_i → Ser + P_i would be −2.4 kcal, *i.e.*, $-RT$ ln 55. Since $\Delta F°_{hyd}$ for serine phosphate is −3.3 kcal (Vladimirova *et al.*, 1961),[12] $\Delta F°$ for bond cleavage, *i.e.*, Ser-P → Ser·P_i would be −0.9 kcal, as indicated in Figure 9B.

The problem of using $\Delta F°$ values for phosphate-bond cleavage in comparing phosphate esters is illustrated by the difference in these values for phosphoglucomutase (−5.3 kcal) and phosphoserine (−0.9 kcal) (see Figure 9). The difference of about 4.4 kcal means that positive noncovalent interactions between the enzyme and phosphate group *increase* when the enzyme–phosphate bond in PGM is broken, *viz.*, changes in noncovalent interactions cause the $\Delta F°$ value for bond breaking to be more negative than that of phosphoserine. Hence to the extent that noncovalent interactions are altered by the bond-breaking step, this step will be an unreliable basis for making comparisons, unless the identity of the bond that is broken is known in advance, and the comparison sought is the extent of alteration in noncovalent interactions. Of course factors such as changes in the conformation of the protein might be involved in altering the $\Delta F°$ value for bond breaking. However, for purposes of simplicity, only interactions between the enzyme and the phosphate moiety are referred to in this discussion. If changes in protein conformation are involved, the arguments can be altered accordingly.

Figure 10 compares free-energy diagrams for a two-step hydrolysis of the protein–phosphate bond in PGM, phosphomonoesterase (Hsu *et al.*, 1966), and alkaline phosphatase (Wilson and Dayan, 1965). Note, however, that the energy level for the phospho form of phosphomonoesterase represents only a minimum value (Hsu *et al.*, 1966) as indicated by the stippling in Figure 10B.

The large positive value of $\Delta F°_{hyd}$ for the phosphate group of alkaline phosphatase, +5.5 kcal, in comparison with the value of −3.3 kcal for phosphoserine suggests the presence of large positive noncovalent interactions between the phosphate group and the protein in the phospho form of the phosphatase, while the relatively small $\Delta F°$ for bond breaking, −2.5 kcal, suggests that these interactions are not extensively altered during bond breaking. By contrast, noncovalent phosphate:protein interactions seem to be much smaller in the phospho form of PGM because of the small negative value of $\Delta F°_{hyd}$, but these interactions apparently increase to a significant degree on cleavage of the enzyme–phosphate bond, as indicated above.

Although data are not available to allow the hydrolysis of a phosphate group in the phosvitin system to be separated into two steps, it seems doubtful that inorganic phosphate would bind to the protein because of the high net negative charge on the protein produced by the presence of other phosphate groups. In such a case, the $\Delta F°$ for bond breaking might well be similar to that of phosphoserine while the $\Delta F°$ for separation of products would be much more negative than that assumed for the comparable step in the hydrolysis of phosphoserine.

The attachment site of the phosphate group in the phosphomonoesterase system is not known and, from the foregoing discussion, cannot be identified from $\Delta F°$ values for phosphate hydrolysis. *If* the site of attach-

ment is the hydroxyl group of a serine residue, a large positive increase in noncovalent interactions, as in PGM, is required to explain the very favorable value of $\Delta F°$ for bond breaking, which is negative by at least −6.2 kcal, and relatively insignificant noncovalent interactions between the phosphate group and the enzyme might well exist in the phosphoenzyme.

Appendix

By applying the King–Altman (1956) procedure to the Ping-Pong reaction of eq 2 and 3 the distribution equations for $[E]/[E_T]$ and $[F]/[E_T]$ may be obtained.

$$[E]/[E_T] = ([B]\text{coef B} + [P]\text{coef P})/\text{den. terms}$$

$$[F]/]E_T] = ([A]\text{coef A} + [Q]\text{coef Q})/\text{den. terms}$$

where coef and den. terms stand, respectively, for coefficient and denominator terms and are defined by Cleland (1963a). By factoring and using Cleland's definitions of constants

$$[E]/[E_T] = [P]\text{coef P}\left(1 + \frac{[B]K_{i(P)}K_A}{[P]K_{i(A)}K_B}\right)\Big/\text{den. terms}$$

$$[F]/[E_T] = [A]\text{coef A}\left(1 + \frac{[Q]K_{i(A)}K_A}{[A]K_{i(B)}K_B}\right)\Big/\text{den. terms}$$

At equilibrium the ratio

$$\left(1 + \frac{[B]K_{i(P)}K_A}{[P]K_{i(A)}K_B}\right)\Big/\left(1 + \frac{[Q]K_{i(Q)}K_A}{[A]K_{i(B)}K_B}\right)$$

is equal to unity since $K_{i(P)}K_{i(Q)}/K_{i(A)}K_{i(B)} = K_e$ (Cleland, 1963a). Hence

$$\frac{[F][P]}{[E][A]} = \frac{\text{coef A}}{\text{coef B}} = \frac{K_{i(P)}}{K_{i(A)}}$$

An analogous procedure gives

$$\frac{[E][Q]}{[F][B]} = \frac{\text{coef Q}}{\text{coef B}} = \frac{K_{i(Q)}}{K_{i(B)}}$$

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