noncovalent interactions increase during this process may be approximated as about −1 kcal/mol, by comparison with the *hypothetical* transfer in process A4, where electrostatic effects have been eliminated by appropriately positioning the PO_3^{2-} group *prior* to transfer and moving it an inappreciable distance during transfer (as is presumably the case in process A3). A somewhat larger value of about 3 kcal/mol can be calculated for the increase in noncovalent interactions during the analogous process involving E_P·Glc-6-P and E_D·Glc-P_2. (The same changes in noncovalent interactions are obtained by comparing values of $\Delta G°'$ for hydrolysis of Ser-P, E_P, E_P·Glc-1-P, and (Ser-P·Glc-1-P): see parameters in the Results and the review by Ray and Peck (1972).)

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An Analysis of the Substrate-Induced Rate Effect in the Phosphoglucomutase System†

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ABSTRACT: The rate constant for the catalytic transfer of the active-site PO_3 group from rabbit muscle phosphoglucomutase to the hydroxyl group of a water molecule is about 3×10^{-8} s^{-1} under optimal reaction conditions, but in the absence of the normal substrate, viz., at pH 7.5 and 30 °C, in the presence of saturating Mg^{2+}; the corresponding constant for transfer to the 6-hydroxyl group of glucose 1-phosphate under analogous conditions, about 1000 s^{-1}, is larger than this by some 3×10^{10}-fold. Since no single factor appears to be capable of providing a rationale for a majority of this "substrate-induced rate effect" (Ray, Jr., W. J., and Long, J. W. (1976), *Biochemistry,* the preceding paper in this issue), the change in the PO_3-transfer rate produced by binding various parts of the phosphoglucosyl moiety to the enzyme, both separately and concurrently, was investigated. The rate of PO_3 transfer to water is increased by up to 1000-fold by binding entities that provide the active site with a second PO_3 group, e.g., ethyl phosphate or inorganic phosphite. Using an alcoholic acceptor further increases transfer efficiency (in the presence of bound phosphite): increase with methanol, about 2000-fold on a molar basis. The reactivities of ten other primary aliphatic alcohols vary by nearly 600-fold as the acidity of the PO_3 acceptor is varied over a 4000-fold range. Although no straightforward relationship is observed between the efficiency of an alcohol as an acceptor and its acidity—presumably because of complications due to steric effects, for example—an increased

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transfer rate of 100-fold, relative to the water reaction, is estimated for a simple primary alcohol with a pK_a similar to that expected for the 6-hydroxyl group of glucose 1-phosphate, when the alcohol is present at a concentration of 1 M. Joining an alcoholic acceptor and a PO_3 group via five apparently inert bridging units changes PO_3 transfer to an intramolecular process; in the case of 1,4-butanediol monophosphate the rate of transfer also increases by 240-fold, relative to the analogous reaction in the presence of 1 M propanol and bound inorganic phosphite. Comparable values also are obtained in comparisons of PO_3 transfer rates for *trans*-1,4-butenediol and 1,4-butynediol monophosphates relative to 1 M allyl and propargyl alcohols, respectively, in the presence of bound phosphite. An increased rate of transfer also is produced by binding the xylosyl part of the glucose ring, either when the acceptor is an hydroxyl group attached to the ring or when it is the hydroxyl group of a water molecule, e.g., as in the water reaction facilitated by bound xylose 1-phosphate. These and other results suggest that most of the difference between the rates of the water reaction and the glucose 1-phosphate reaction can be rationalized in terms of four fairly discrete factors whose approximate values are as follows: the PO_4 factor, 1000-fold; the C-OH/H-OH factor, 100-fold; the nucleophile-binding factor, 250-fold; and the $(CHOH)_3$-bridging factor, 200-fold. The intrinsic binding energy [cf. Jencks, W. P. (1975), *Adv. Enzymol. 43,* 219] of glucose 1-phosphate, approximated as the sum of the observed Gibbs binding energy for glucose 1-phosphate plus the difference in Gibbs activation energies for PO_3 transfer to water and to glucose 1-phosphate, must be in the neighborhood of −20 kcal/mol. The dianionic phosphoester group supplies about half of this binding energy. Possible ways in which the intrinsic binding energy that can be associated with the glucose 1-phosphate molecule might be used to elicit the substrate-induced rate effect are considered in light of the reactivity of a number of other acceptors and acceptor–activator pairs.

The rates of many enzymatic reactions are much slower with substrates that are smaller than the natural substrate, and two different types of explanations were advanced a number of years ago to rationalize such differences: the induced-fit and nonproductive complex hypotheses of enzyme specificity (Koshland, 1963; Hien and Nieman, 1961). More recently, along with the increased appreciation of the subtle interplay that exists between binding interactions and catalysis, has come the realization that smaller-than-nornal substrates may simply lack the "intrinsic binding energy" (cf. Jencks, 1975) to react as readily as the normal substrate. This is certainly a valid explanation for most of the huge difference in the rate of PO_3 transfer from phosphoglucomutase to the 6-hydroxyl group of bound glucose-1-P[1] and to the chemically similar hydroxyl group of water (see Results); however, it is an ambiguous explanation from a mechanistic standpoint. Thus, there are several different ways in which substrate binding energy might be used to reduce the Gibbs activation energy for the catalytic process: e.g., to produce either enthalpic destabilization or (entropic) immobilization of the reactant groups in the enzyme–substrate complex, or to produce transition-state stabilization by means of an arrangement to allow increased binding of nonreacting groups to partially compensate for the energy required for breaking chemical bonds. In fact, each of these three possibilities, in theory, appears to be capable, by itself, of providing a rationale for large majority of the huge (3×10^{10}-fold) substrate-induced rate effect in the present system, and each was considered separately in an accompanying paper (Ray and Long, 1976a). However, none accounts for anything like a majority of this rate effect, and several different effects must operate together to produce it. The present study was undertaken in an attempt to define the origin of some of these factors by examining the efficiency of PO_3 transfer from the phospho-enzyme to various acceptors as a function of their structure—particularly acceptors intermediate in complexity between water and glucose phosphate.

Materials and Methods

Procedures for isolating phosphoglucomutase, determining its concentration, converting it to the Mg^{2+} complex, and labeling its active-site serine with ^{32}P-labeled phosphate have been referred to in this series (Ray and Long, 1976a,b). However, overnight dialysis against 1 mM EDTA–20 mM Tris-Cl, pH 7.5, was included as the final step in the latter procedure in order to further reduce Cl_3COOH-soluble radioactivity associated with the labeled enzyme used in some of the experiments reported here. Procedures for determining the rate at which the ^{32}P-labeled phosphate group of the enzyme was transferred to an acceptor were analogous to those described previously (Long and Ray, 1973); however, when the extent of transfer in time aliquots of reaction mixtures was less than 5%, the Cl_3COOH-soluble radioactivity was subjected to additional protein precipitation steps by (a) adding aliquots of bovine albumin that contained inorganic phosphate and (b) centrifuging, after cooling at 0 °C for 20 min. For transfer reactions of greater than 24 h duration, solution components were "sterilized" by passage through a 0.45 μ Millipore filter. Because these filters absorb significant amounts of phosphoglucomutase, an analogous solution containing several milligrams of unlabeled enzyme was passed through a filter and discarded before the labeled enzyme was filtered. This allowed essentially all of the radioactive enzyme to be recovered in the filtrate. Such long-term reactions also were conducted in sterile, 1.5-ml Eppendorf centrifuge tubes which were closed and placed in a water-tight container; the container was completely immersed in a water bath at the desired temperature. At the end of such reactions, samples were plated on a "rich" agar medium and incubated for several days to check for bacterial contamination. Transfer reactions with half-lives of 2 to 20 s were conducted in a flat-bottomed "mini beaker" held in an aluminum block atop a sealed magnetic stirrer; the block and stirrer were immersed in a water bath to provide temperature control. The reaction was initiated by rapid addition of a small volume of acceptor, previously brought to the temperature of the bath, to a much larger volume of a rapidly stirred reaction mixture, also at bath temperature, which contained the labeled enzyme; the reaction was followed either by removing time aliquots and assaying for Cl_3COOH-soluble radioactivity, as above, or by using the entire reaction mixture for a single time point. No special precautions were taken during initiation of transfer reactions with intermediate rates; usually these were followed by means of the former sampling procedure. Most reactions were fol-

[1] The following abbreviation is used: -P, a PO_3 group, usually of a phosphate ester, in the dianionic form unless otherwise specified (thus methyl phosphate is CH_3OP, inorganic phosphite is HP and α-D-glucose 1-phosphate is Glc-1-P); Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance.

lowed for between 1 and 3 half-times and rate constants were estimated from semilog plots of the fraction of labeled enzyme remaining vs. time. Such plots always involved at least three and usually five or six points. Individual points frequently were run in duplicate and the results averaged.

Labeled inorganic phosphate was determined by measuring the radioactivity in aliquots before and after precipitating with triethylamine molybdate; acid-labile organic phosphate was determined by heating in 1 N acid for 10 min at 100 °C and subjecting the product to a precipitation step as above, while acid-stable organic phosphate was calculated from the radioactivity remaining in the supernatant subsequent to these steps (cf. Peck et al., 1968; however, two centrifugations in an Eppendorf microcentrifuge, the second in a clean tube, were substituted for the filtration step used by these authors).

α,ω-Diol monophosphates were prepared from the corresponding crude diol monoacetates (see below) by the method of Tenner (1961), which involves coupling with excess cyanoethyl phosphate in pyridine in the presence of excess dicyclohexylcarbodiimide (an overnight reaction at room temperature with a final concentration of about 0.1 M diol monoacetate usually was used), followed by a basic hydrolysis step. (In the case of *trans*-butanediol-P and 2,2,3,3-tetrafluorobutanediol-P, yields were improved by isolating the phosphomonoester via anion-exchange chromatography prior to the hydrolysis step.) When possible, the hydrolysis was conducted in 0.2 M NaOH at 100 °C for 15 min in order to hydrolyze unreacted cyanoethyl phosphate; otherwise, 0.1 N NaOH at 50 °C for 5 min was used and the unchanged cyanoethyl phosphate was separated in the subsequent step. (Cyanoethyl phosphate in chromatographic fractions is easily identified and quantitated by taking advantage of its base lability (see above); however, the hydrolysis step must be conducted in plastic test tubes to avoid formation of silicates.) Gradient elution chromatography on a column of a Dowex-1 resin, formate or bicarbonate form, was used to isolate the products (cf. Ray and Roscelli, 1964). The phosphates of simple alcohols were prepared in a similar manner from the free alcohol. Yields usually were about 40%, but were much lower for *trans*-butenediol-P, butynediol-P, and 2,2,3,3-tetrafluorobutanediol-P. In these cases yields were improved by using a 2-h reaction at twice the concentration of reactants noted above, as well as by employing the milder hydrolysis procedure. The identity of each product was confirmed by its proton NMR spectrum, which was obtained in D_2O, either with a 60 or a 100 megacycle instrument. Most diol monoacetates used in the above reactions were prepared by reaction of the diol with an equimolar amount of acetic anhydride in pyridine. In the case of *trans*-butenediol, the diacetate, prepared from the corresponding dichloride (Eastman) according to Thomas and Warburton (1965), was equilibrated for 48 h with 1 equiv of dry methanol containing 0.03 equiv of sodium methoxide. The monoacetates were partially purified by (a) evaporating most of the solvent at reduced pressure in a rotary evaporator, (b) adding water (in the case of *trans*-butenediol monoacetate, the reaction mixture also was neutralized at this point) and extracting the bisacetate with CCl_4, (c) saturating the water layer with $CaCl_2$ and extracting the monoacetate with $CHCl_3$, and (d) evaporating the $CHCl_3$ under reduced pressure after drying it over $CaCl_2$. All diols used as starting materials were commercially available except for 2,2,3,3-tetrafluorobutanediol, which was prepared from the corresponding diethyl ester of tetrafluorosuccinic acid (PRC) by $LiAlH_4$ reduction (McBee et al., 1952). (The *cis*-butenediol was "91% cis isomer"; however, the trans isomer present was unimportant since the yield of *trans*-2-butenediol-P in the cyanoethylation step was negligible under the conditions used to prepare the corresponding *cis*-diol-P.)

1-Hydroxymethyl-2-oxa-1,4-butanediol-1-P and 1,3-bis(hydroxymethyl)-2-oxa-1,4-butanediol-1-P were prepared from xylose-1-P and glucose-1-P, respectively, by (a) oxidizing a 10 mM solution of the sugar phosphate for 20 h at pH 4 and 30 °C with 3 mol equiv of KIO_4 (cf. Dryhurst, 1970), (b) reducing the product with excess $NaBH_4$ after adjusting the pH to 8 with NaOH, (c) neutralizing with Dowex-50-H^+ and degassing the filtered solution under reduced pressure, and (d) chromatographing on Dowex-1-HCO_3 by using a triethylammonium bicarbonate elution gradient. Since both compounds are rapidly hydrolyzed under the conditions of the Fiske–SubbaRao procedure for phosphate analysis (cf. Leloir and Cardini, 1957), the essential absence of inorganic phosphate (and thus the lack of spontaneous hydrolysis) in the final product was checked by comparing the phosphate content of a stock solution before and after a precipitation step with $BaCl_2$ at pH 8 under conditions where inorganic phosphate equivalent to 1% of the total phosphate present formed a precipitate. (Hydrolysis equivalent to 10 to 20% of the starting material was observed in step a.)

D-Sorbitol-6-P and D-erythritol-4-P were prepared from D-glucose-6-P and D-erythrose-4-P, respectively, by $NaBH_4$ reduction in near neutral aqueous solution, followed by a chromatographic isolation step. 6-Deoxyglucose-1-P was prepared from the corresponding deoxy sugar (secured as a gift from the Atlas Powder Co.) by the procedure of Hanna and Mendicino (1970). Any glucose-1-P present after ion-exchange chromatography of the product was removed by treatment with glucose-6-P dehydrogenase and $NADP^+$ in the presence of small amounts of phosphoglucomutase, in the manner described previously for purifying xylose-1-P (Ray and Long, 1976a); a second chromatographic separation subsequently was performed. Glucose 6-sulfate was prepared as described previously (Peck et al., 1968). Solutions of glucose (4 M) were purified in the manner described for purifying xylose (Ray and Long, 1976a). α,D-Glucose-1-P, α,D-xylose-1-P, D-erythrose-4-P, and DL-α-glycero-P were obtained from Sigma and were used without further purification. The best commercial grades of all other chemicals were used.

Results

Because phosphoglucomutase requires a bivalent metal ion for maximal activity (cf. Ray and Peck, 1972) all of the studies reported here were conducted either in the presence of saturating Mg^{2+} or, in the case of controls, in the absence of any bivalent metal ion. Hence, all references to the enzyme will imply the Mg^{2+} complex unless otherwise indicated.

PO_3 Transfer to Water and the Effect of Phosphate Derivatives on the Rate of Transfer. Under optimal assay conditions (1 mM Mg^{2+}, 20 mM Tris-Cl, pH 7.5), but in the absence of substrate, [^{32}P]phosphoglucomutase undergoes an extremely slow reaction as is shown in Figure 1 by the increase in Cl_3CCOOH-soluble radioactivity with time (initial Cl_3CCOOH solubility: 0.24 ± 0.02%). Essentially all of this increase can be accounted for in terms of inorganic phosphate, and the reaction thus represents the slow transfer of the enzymic PO_3 group to water (see below). Although the enzyme was otherwise quite stable under these conditions (the activity measured in the standard assay decreased by only a few percent during the time intervals used), we were unable to follow the Figure 1 reaction long enough to establish that it was indeed first order (the half-time at 30 °C would have been about 8

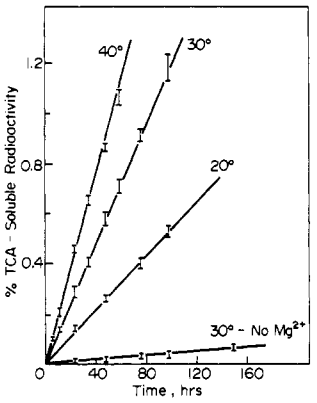


FIGURE 1: The increase in Cl_3CCOOH-soluble radioactivity with time on exposure of ^{32}P-labeled phosphoglucomutase to 20 mM Tris, pH 7.5. The reaction, which is described in greater detail in the Materials and Methods section, was conducted in three separate vessels at *each temperature;* three aliquots of each of the three reaction mixtures were removed at the indicated time intervals. Each aliquot was subjected to three sequential Cl_3CCOOH precipitation steps and the soluble radioactivity after the third precipitation was measured. The error bars show the standard deviation calculated from these nine measurements. The Cl_3CCOOH-soluble radioactivity at zero time was 0.24 ± 0.02%, and the increase over the initial value is shown.

TABLE I: Rates of PO_3-Transfer from Phosphoglucomutase to Water or Methanol in the Presence of Anionic Activators.[a]

PO_3 Activator	Relative Rate[b]	
	Water	Methanol (1 M)
None	1.0[c]	10
Phosphite	650	20 000[d]
Phosphate	50	3 500[d]
Methanol-P	450	
Ethanol-P	1000	
Ethylene glycol-P	500	
Propanol-P	750	
Propanediol-P	5000	800[e]
α-DL-Glycerol-P	4600	
α-D-Xylose-1-P	2×10^5	<200[f]
6-Deoxyglucose-1-P	<13[g]	

[a] At pH 7.5 and 30 °C. [b] Relative first-order rate constant for appearance of Cl_3CCOOH-soluble radioactivity from [^{32}P]phosphoglucomutase; the radioactivity released both as inorganic and organic phosphate was measured separately. [c] Actual rate = 3.2×10^{-8} s^{-1} (see Results). [d] Calculated as $r/[MeOH]$, where r is the ratio of the methyl phosphate to inorganic phosphate obtained from a reaction in dilute methanol solution. [e] Calculated as in *d* but from a reaction conducted with 4 M methanol. [f] No methyl phosphate detected. [g] The actual value may be substantially less than that shown.

months). However the rate constant for PO_3 transfer to water was calculated on this basis—$k = (3.2 \pm 0.2) \times 10^{-8}$ s—since a variety of simple activators (see below) increase the rate of PO_3 transfer to water sufficiently to verify a first-order transfer. Similar rate constants for the water reaction were obtained with samples of enzyme from three separate preparations.[2]

Even though the water reaction is quite slow, at least part of the catalytic apparatus of the enzyme must be involved in this reaction since (a) the rate is about 300-fold faster than the extrapolated value for the hydrolysis of serine phosphate at pH 7.5 (cf. Benkovic and Schray, 1973), (b) the effect of temperature on the transfer rate (see below) is much smaller than for the nonenzymic hydrolysis of a variety of phosphate ester at neutral pH (Kosower, 1962), (c) the reaction rate is decreased by at least 30-fold in the absence of an activating metal ion (see Figure 1), and (d) in the presence of 2 M methanol most of the product is methyl phosphate (see below), thus ruling out a base-catalyzed β-elimination of phosphate (cf. Samuel and Silver, 1963). Of course, since the extent of the reaction at most was only slightly in excess of 1%, it is not possible to prove that the inorganic phosphate produced did not involve the decomposition of an impurity (see footnote 7). However, this would not substantially alter our conclusions; it would simply make the disparity between the rates of the glucose-1-P reaction and the true water reaction somewhat greater. That the water reaction does not involve the action of a nonspecific phosphatase contaminant on labeled phosphoglucomutase is indicated by (a) the studies of Kirkpatrick (1967) who showed that the rate constant for the reaction was independent of the protein concentration used over a wide range (which would require that the hypothetical phosphatase have an inordinately high affinity for the phospho-enzyme), (b) the increase (as opposed to a decrease) in the rate of the water reaction that was observed on addition of inorganic phosphate and phosphate derivatives (see below), (c) the stability of glucose phosphates in the presence of a tenfold excess of phosphoglucomutase over a 24-h time period (see Ray and Long, 1976a).

Table I shows rate constants for PO_3 transfer from the phospho-enzyme to water under conditions analogous to those described above, but in the presence of several PO_3-containing derivatives that resemble parts of the normal substrates. These vary in size from inorganic phosphite to xylose-1-P and 6-deoxyglucose-1-P. Other experiments in which the effector concentration was varied (not shown) demonstrated that the concentrations used to obtain the data in Table I produced at least 90% of the maximum transfer rate and that at least 98% of the radioactive product was inorganic phosphate (see Materials and Methods).

PO_3 Transfer to Alcohols. Table I also shows the PO_3-transfer rates observed in the presence of methanol, alone, as well as in the presence of some of the above activators. In these as well as other reactions involving simple alcohols (see below), the ratio of organic phosphate to inorganic phosphate that was produced was constant throughout the reaction, as expected if water and alcohol compete as acceptors for the enzymic PO_3 group. In the case of methanol plus phosphite, the radioactive products were separated by means of ion-exchange chromatography; the profile of the eluted radioactivity was coincident with the concentration profile produced by inorganic phosphate and methyl phosphate, which were added as carriers prior to the chromatographic separation (not shown).

A number of different primary alcohols were investigated as PO_3 acceptors in the presence of saturating phosphite. In the case of methanol, the transfer rate was proportional to concentration in the range 0.02 to 2 M alcohol, although in the range 2 to 10 M alcohol the rate increased more rapidly than expected from the results obtained at the lower concentrations. Moreover, at 2 M methanol, the rate constant for the water reaction also had increased by about twofold over that in the presence of phosphite, alone, as assessed by an ion-exchange

[2] Kirkpatrick (1967) obtained a value of 3×10^{-7} s^{-1} for the above hydrolysis constant but conducted the reaction in the presence of 0.9 mM inorganic phosphate and 2 mM inorganic sulfate; a similar rate constant under comparable conditions also was obtained in this study.

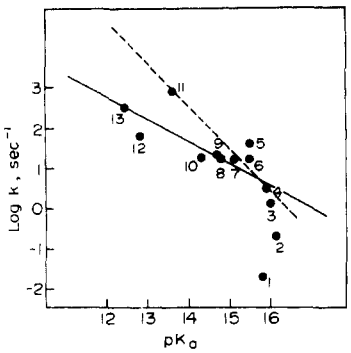


FIGURE 2: A plot of log k for PO_3 transfer from phosphoglucomutase to the indicated alcohol at pH 7.5 and 30 °C in the presence of 40 mM (saturating) sodium phosphite and 20 mM Tris-Cl vs. the pK_a of the acceptor alcohol. ^{32}P-labeled phosphoglucomutase was used; values of k were calculated as $fk^{app}/[ROH]$; see the Results. The numbers refer to the acceptor used: 1, water (k calculated on the basis of a 1 M concentration); 2, 2-propanol; 3, 1-propanol; 4, ethanol; 5, methanol; 6, allyl alcohol; 7, ethylene glycol; 8, ethylene glycol monomethyl ether; 9, glycerol; 10, chloroethanol; 11, propargyl alcohol; 12, 2,2,4,4-tetrafluoropropanol; 13, trifluoroethanol. pK_a values were taken from Ballinger and Long (1960); measured pK_a values for glycerol and ethylene glycol were corrected for the statistical factor. The solid line represents the best linear correlation for all of the data points, excluding those for acceptors 1, 2, and 11; the dashed line shows a similar correlation for acceptors 3, 6, and 11, only (see Results).

TABLE II: Kinetic Constants for PO_3 Transfer from Phosphoglucomutase to α,ω-Diol Monophosphates.[a]

Acceptor	$10^6 \times$ Relative k[b]	K_d[c] (mM^{-1})
1,4-Butanediol-P	0.20[d]	4
cis-2-Butene-1,4-diol-P	0.44	1.0
trans-2-Butene-1,4-diol-P	1.4	2.0
2-Butyne-1,4-diol-P	47	2.2
D-Erythritol-4-P	0.24	
1-Hydroxymethyl-2-oxabutane-1,4-diol-P	0.9[d]	3.2
1,3-Bishydroxymethyl-2-oxabutane-1,4-diol-P	0.09	5
1,5-Pentanediol-P	0.013	
Sorbitol-6-P	0.0011	

[a] At pH 7.5 and 30 °C. [b] Maximum values of the pseudo-first-order rate constant for PO_3 transfer were obtained from double-reciprocal plots of observed rate constants (using [^{32}P]phosphoglucomutase; see Materials and Methods) and acceptor concentration and rates calculated with respect to a rate constant of 3.2×10^{-8} s^{-1} for the water reaction (see Results). [c] Values of K_d were taken as equal to the corresponding K_m values; see *b*. [d] Values verified by steady-state assays (see Results).

chromatographic separation of the methyl phosphate and inorganic phosphate produced during a given time interval. With the much less efficient 1-propanol, an increase in the water reaction of 1.6-fold was observed at a concentration of 1 M alcohol by direct measurement of the rate at which labeled inorganic phosphate was produced. These anomalous increases apparently were caused by a sort of general medium effect since added dimethyl sulfoxide also increased the rate of the water reaction—and increased the rate of the methanol reaction (at constant methanol) to a similar extent. In order to eliminate as much as possible of this medium effect, all alcohols were used at concentrations of 0.1 M or less, except for the propyl alcohols, which were used at 0.5 M. In addition, the rates of both the water and alcohol reactions were followed simultaneously, and in most cases the concentration of alcohol was adjusted in successive studies so that in the second study approximately 50% of the reaction was with water. Under these conditions the rate constant for the water reaction was unchanged at 2.1×10^{-5} s^{-1} and a general solvent effect thus was not involved, except when the propanols were used (see below). Rate constants were calculated as $fk^{app}/[ROH]$, where k^{app} is the pseudo-first-order rate constant for disappearance of [^{32}P]E_P and f is the fraction of the label released as organic phosphate. In the case of ethylene glycol and glycerol the effective concentration was taken as half the molar concentration. In the case of the propyl alcohols a direct correction for the medium effect was made by assuming that both the water reaction and the alcohol reaction were similarly altered. The logarithms of the various rate constants are given in Figure 2 as a function of the pK_a of the acceptor alcohol. Although steric problems and desolvation effects (cf. Jencks and Gilchrist, 1962) as well as the possibility that some alcohols bind weakly to the enzyme prevent a straightforward interpretation of this plot (see Discussion), in general, acceptor efficiency increases with decreasing pK_a.

PO_3 Transfer to Bound Diol Monophosphates. The smallest hydroxylic *acceptors* that bind sufficiently well for saturation effects to be demonstrated are the C_4-diol monophosphates. Thus, the smaller $HO(CH_2)_3OP$ acts as an *activator* of the water reaction, as opposed to an acceptor, and at least 98% of the PO_3 transfer in the presence of saturating $HO(CH_2)_3OP$ is to water (no labeled organic phosphate detected); in the presence of 4 M methanol, approximately 40% of the reaction is with the added methanol and the rest with water (see Table I). By contrast, less than 2% of the reaction is with water (no inorganic phosphate detected) when a saturating concentration of 1,4-butanediol-P (20 mM) is used and in the presence of 4 M methanol no methyl phosphate was detected. The rate of PO_3 transfer at saturating concentrations of the various diol monophosphates used, relative to the rate of the water reaction, as well as the dissociation constants for enzyme–acceptor complexes, both of which were obtained from double-reciprocal plots of transfer rate and acceptor concentration (not shown), are given in Table II. In the case of $HO(CH_2)_4OP$ and $HOCH_2C{\equiv}CCH_2OP$, the rate of the transfer process also was measured by means of a steady-state assay analogous to that described in an accompanying paper for the xylose-1-P reaction (Ray and Long, 1976a). In both cases these rates were essentially the same as the corresponding rates obtained in "single turnover" experiments with labeled enzyme.

PO_3 Transfer to Glucose, Xylose, and Glucose 6-Sulfate. The reaction of xylose with phosphoglucomutase produces a product with an acid-labile phosphate that co-chromatographs with α-D-xylose-1-P in an ion-exchange system that separates xylose-1-P from glucose-1-P (Owens, 1971). The rate of the reaction as a function of xylose concentration is shown as a double-reciprocal plot in an accompanying paper (Ray and Long, 1976a). In the case of glucose, the product was almost entirely acid stable, and was presumed to be glucose-6-P. Since the interconversion of glucose-1-P and glucose-6-P is much faster than the rate of PO_3 transfer to free glucose, an initial transfer to the 1-position of the glucose molecule cannot be ruled out, but seems unlikely. Unfortunately, a double-reciprocal plot of transfer rate and glucose concentration begins to curve downward at concentrations greater than about 0.5 M (not shown). Presumably this effect is analogous to the medium effect observed with smaller alcohols (see above), although it is not obvious why a similar effect either was absent or much

less important in the xylose reaction.[3] Because of this problem, the kinetic constants for the glucose reaction are only approximations obtained by using rates measured in the linear portion of the double-reciprocal plot, i.e., at concentrations of glucose substantially below the value of its Michaelis constant. These estimates, together with the constants for the xylose reaction, are given in Table II.

The glucose reaction also was studied in the presence of 25 mM inorganic phosphate under the same conditions as the reaction with glucose, alone. Although a less thorough substrate–velocity study was conducted, a double-reciprocal plot of initial velocity and glucose concentration appeared to be linear in the range 0.2 to 1 M; however, this plot (not shown) also appeared to pass through the origin and a Michaelis constant greater than 10 M was estimated. The pseudo first order rate constant for the reaction at 1 M glucose was 0.09 s^{-1}. A comparable rate constant also was observed with 1 M xylose in the presence of 25 mM phosphate: 0.10 s^{-1}.

The reaction of glucose 6-sulfate with phosphoglucomutase produces the dephospho-enzyme and a mixed phosphate–sulfate ester of glucose (Peck et al., 1968). In the presence of excess glucose-1,6-P_2 a mixture also containing glucose-6-P is produced. The initial rate of the latter reaction was measured by using uniformly labeled [^{32}P]glucose-1,6-P_2 in an assay analogous to that used for the xylose plus phosphite reaction, except that only one of the labeled products ([^{32}P]glucose-6-P) was separated from the labeled reactant by means of the chromatographic procedure described in an accompanying paper (Ray and Long, 1976a). Double-reciprocal plots of velocity and glucose 6-sulfate concentration produced values of 120 s^{-1} and 40 mM for V_{max} and K_m, respectively.

Temperature Effects. The effect of temperature on the rates of several transfer reactions in which the activator or the acceptor can be used at a saturating concentration is shown in Figure 3. In all cases Tris buffer with a pH of 7.5 at 25 °C was used throughout (since the rate of the normal enzymic reaction is insensitive to pH effects in the neutral range (Ray et al., 1966)). Because of the large differences in absolute rate, plots were scaled in the manner indicated in the figure. Although it would be difficult to interpret the small differences in the apparent values of the activation energy for these reactions, the plots at least show that the *relative* values of the rate constant for PO_3 transfer in the various processes are not substantially temperature dependent.

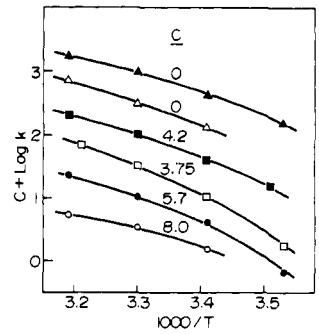


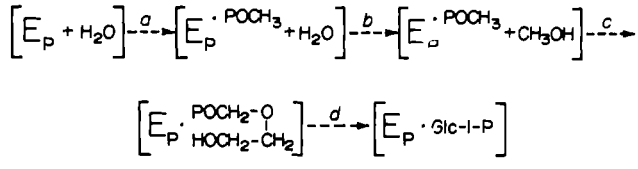
FIGURE 3: Plots of log k for PO_3-transfer reactions of phosphoglucomutase vs. the reciprocal of the temperature at "pH 7.5". Reactions were conducted in 20 mM Tris buffer that was adjusted to pH 7.5 at 25 °C; saturating concentrations of activators or of PO_3 acceptors were maintained. The PO_3 acceptors involved and the reactions monitored are as follows: (▲) Glc-1-P, conversion of Glc-1-P to Glc-6-P; (△) Glc-6-P, conversion of Glc-6-P to Glc-1-P; (■) water, PO_3 transfer in the presence of bound xylose-1-P; (□) 1,4-butanediol-P, PO_3 transfer to the diol-P; (●) water, PO_3 transfer in the presence of bound inorganic phosphite; (○) water, PO_3 transfer in the absence of activators.

Discussion

The huge difference of about 3×10^{10}-fold in the rate at which phosphoglucomutase transfers its PO_3 group to the 6-hydroxyl group of the natural substrate, glucose-1-P, and the rate at which it transfers the same group to water (see Results), *viz.*, the substrate-induced rate effect, is equivalent to a difference in Gibbs activation energies of some 14 kcal/mol. In a recent review, Jencks (1975) pointed out that this rate increment ultimately must depend on binding interactions involving the nonreacting phosphoglucosyl moiety—whether binding induces a catalytically active form of the enzyme, immobilizes, or destabilizes the reactant groups, stabilizes the transition state, etc. However, evidence presented in an accompanying paper (Ray and Long, 1976a) suggests that neither enthalpic destabilization nor entropic immobilization of the reactant groups, nor increased binding interactions of the phosphoglucosyl moiety in the transition state, when taken individually, can account for a major fraction of the substrate-induced rate effect. Hence, in order to define in a more precise manner *how* binding interactions are used to produce the substrate-induced rate effect, the rate of transfer of the enzymic PO_3 group to a variety of structurally different acceptors has been examined as a function of the structure of the acceptor, and in some cases of the accompanying activator.

The approach employed involves making a series of stepwise changes to convert the enzyme–water system into the enzyme·glucose-1-P system and evaluating the effect of each change on the rate of the corresponding PO_3-transfer process. Scheme I shows a possible sequence of this type; it suggests that an effect on the rate of the catalytic PO_3 transfer might be produced by (a) binding a phosphate ester group, (b) using an alcohol as an acceptor instead of water, (c) joining the phosphate group and the acceptor via a chemical bridge so that the transfer becomes an intramolecular process, and (d) adding the $(CHOH)_3$ portion of the glucose ring.

SCHEME I. A Series of Stepwise Changes by Means of Which the Water Reaction Can Be Related to the Glucose-1-P Reaction.[a]



[a] The letter above each arrow serves to relate the indicated change to an increased efficiency of PO_3 transfer produced by that change (see text).

Unfortunately, the sequence in Scheme I presents several mechanistic problems. For example, in the system produced by the step c bridging process, the pK_a of the acceptor is substantially decreased, relative to methanol, whereas, from a mechanistic standpoint the entire change in the chemical character of the acceptor ideally should be included in step b. Moreover, numerous attempts to synthesize the diol monophosphate, $HOCH_2CH_2$–O–CH_2OP, were unsuccessful. In addition, regardless of the specific sequence used, if the results are to be unambiguously interpreted, it is necessary that the

[3] A downward curvature in the double-reciprocal plot for the xylose reaction cannot be completely ruled out; however, if present, such curvature is slight, and is completely eliminated by addition of saturating phosphite (see Figure 2 of Ray and Long, 1976a).

rate factors associated with the various steps, e.g., the factor associated with the change from water to methanol as an acceptor (step b) and that associated with the binding of methyl phosphate (step a), be independent of which step comes first in the overall sequence. Although such an independence appears to hold as a first approximation, reversing the above two steps does give rise to differences in the rate changes associated with these steps (see Results). If the overall substrate-induced rate effect were small, these differences might be sufficiently large to discourage an approach of the type taken here. However a difference of twofold, for example, in alternative approximations of the same factor is inappreciable in comparison with the 3×10^{10}-fold rate effect under consideration—at least at the level of sophistication sought here. Because of these problems, the effects produced by the types of changes that were suggested above, based on the sequence in Scheme I, were evaluated in as many different ways as possible. Moreover, in some cases the maximum value within a group of similar values was used, in order to reduce the probability of overestimating factor d for the final step in the sequence, since factor d will be equal to the ratio of the overall effect to the product of factors a, b, and c.

The PO_4 Factor. In Scheme I, methyl phosphate is bound to the enzyme in the first step of the proposed sequence. In fact, a variety of PO_3-containing entities produce similar effects on the rate of transfer of the enzymic PO_3 group to water of up to 1000-fold (see Table I). However, inorganic phosphate produces only a 50-fold increase. Presumably its un-ionized hydroxyl group interacts with a nearby group of the enzyme in a manner that is unfavorable for the transfer process.[4] Hence, the rate factor for inorganic phosphate was ignored in assessing the effect of a bound phosphate ester group on the reactivity of the enzymic phosphate, although this factor is referred to as the PO_4 factor. In addition, the largest rate effect observed, viz., 1000-fold, with ethyl phosphate, was used as the PO_4 factor. However, since inorganic phosphite provides a rate effect similar to that of the above phosphate esters (viz., 650-fold), it was used in many subsequent studies, instead of ethyl phosphate, to minimize possible steric problems (see below).

The C-OH/H-OH Factor. It is more difficult to evaluate the size of the effect involved in the change from water to an alcoholic acceptor with properties similar to those of the 6-hydroxyl group of glucose-1-P. Thus, the relative reactivities of a group of primary aliphatic alcohols, which were tested as PO_3 acceptors (in the presence of bound inorganic phosphite[5]), varied by nearly 600-fold, and the least reactive alcohol was more than 70-fold as reactive as water, on a molar basis. The Bronsted plot in Figure 2, constructed with the data thus obtained, serves as a guide for estimating how chemical properties of the 6-hydroxyl group of glucose-1-P that are different from those of water might influence the transfer process. Clearly, there is no simple relationship between pK_a and acceptor efficiency since, for example, only a minor fraction of the nearly 2000-fold rate difference between methanol and water (on a molar basis) can be produced by pK_a differences. However, within a series of primary aliphatic alcohols, increased reactivity generally accompanies a decreased pK_a and the observed deviations from a simple relationship probably arise from factors such as steric differences, differences in desolvation energy and, possibly, weak binding interactions in either the ground or the transition states, or both. The importance of steric effects is shown by the observation that attaching methyl groups to the methanol moiety, as in ethanol, 1-propanol, and 2-propanol, substantially diminishes the rate constant for the transfer process (by factors of 0.08, 0.03, and 0.005, respectively; Figure 2),[6] although the normal acceptors are much more bulky alcohols, and although there seems to be little difference in the rate of transfer to glucose-1-P, a primary alcohol, and to glucose-6-P, a secondary alcohol (however, see below). Nevertheless, three simple C_3 alcohols with similar overall steric requirements, 1-propanol, allyl alcohol, and propargyl alcohol, produce an essentially linear plot with a slope of about 1 (dashed line), although the slope of the best line through all of the points except those for propargyl alcohol, methanol, and 2-propanol is about 0.5 (solid line).

These observations suggest that the effect of replacing water with an alcohol whose properties are analogous to those of the 6-hydroxyl group of bound glucose-1-P can best be approximated by using an alcohol that is (a) small enough not to pose a steric problem in the displacement process at the enzyme phosphate and (b) one that has a pK_a similar to that of the hydroxyl group in question. Although the pK_a of the 6-hydroxyl group of glucose-1-P has not been measured, its pK_a should resemble that of ethylene glycol methyl ether, 14.8 (Ballinger and Long, 1960), if only inductive effects are important. However, since the transfer process involving the alcohols in Figure 2 apparently is quite sensitive to steric effects (see above) ethylene glycol methyl ether may well be too bulky for the comparison in question, and it seems more reasonable to use methanol as the model alcohol and to include a minor correction for the difference between its pK_a, 15.5, and that expected for the 6-hydroxyl group of glucose-1-P, 14.8 (see above). Thus, if the slope of the appropriate Bronsted plot were 1.0, this difference would amount to a 5-fold rate increase, but only a 2.2-fold increase if the slope were 0.5 (cf. Figure 2). If the more conservative estimate is used, an approximately 100-fold rate increase is expected for the change from water (55 M) as an acceptor to an alcohol with minimal steric requirements and the appropriate pK_a, when it is present at a concentration of 1 M. Hence, the C-OH/H-OH factor will be taken as 100. Although the concentration of 1 M is completely arbitrary, this convention is consistent with that subsequently used for evaluating the nucleophile-binding factor (see below). (Note that this approach, which attempts to optimize the accessibility of the acceptor to the enzymic phosphate at this stage in the Scheme I sequence, by using methanol as the reference alcohol, in fact shifts the burden of eliciting a comparable "accessibility" for the 6-hydroxyl group of the much more bulky glucose-1-P molecule to a later step in the Scheme I sequence (see below).)

The Nucleophile-Binding Factor. Step c of Scheme I represents a change from an external nucleophile (at a concentration of 1 M) to a bound nucleophile, or from an intermolecular to an intramolecular displacement. In general, such a change could alter the rate of PO_3 transfer by means of effects

[4] An interaction with a smaller-than-normal substrate that inhibits catalysis in a manner that apparently is similar to the type of interaction suggested here occurs in the complex of glycyltyrosine and carboxypeptidase (Lipscomb and Hartsuck, 1971).

[5] Phosphite was used as an activator in these studies, instead of phosphate esters, in order to minimize the possibility of steric problems. Thus, propanediol-P increases the rate of the water reaction by 5000-fold but increases the rate of the methanol reaction by only 80-fold, presumably because of a steric problem; by contrast inorganic phosphite produces a similar rate increase in both cases (see Table I).

[6] Moderate steric effects also have been observed in the animolysis of the simple ester, nitrophenyl phosphate (Kirby and Jencks, 1965); hence, the steric effect observed here could be caused partly by the nature of the phosphate group itself and partly by its environment in the enzyme.

that are predominately enthalpic in nature, or predominately entropic, or mixed. However, an accompanying paper (Ray and Long, 1976a) supports the position that substrate binding in the phosphoglucomutase reaction does not produce a substantial destabilization of the enzymic phosphate or acceptor hydroxyl groups, which is the only obvious enthalpic effect that one might expect on changing from a "free" to a bound nucleophile. Because of this, it seems reasonable to identify the rate effect produced by anchoring the nucleophile to the enzyme via a "flexible" bridge attached to the activating PO_4 group (Scheme I), step c, as the nucleophile-binding factor. To avoid substantial enthalpic effects, this bridge should allow, but not force, both the attacking hydroxyl group and the activating PO_4 group to interact with the enzyme during approach to the transition state in the same way that these groups interact with the enzyme during the analogous process in the absence of the chemical bridge. To provide support for defining the nucleophile-binding effect in this manner, the reactivities of a number of alcohols (1 M concentration) toward the enzyme, in the presence of bound inorganic phosphite, were compared with the reactivities of structurally similar diol monophosphates, e.g., $HOCH_2CH_2CH_3$ + HP was compared with $HOCH_2CH_2CH_2CH_2OP$, see Table II. However, even with the information thus obtained it is not easy to demonstrate that the above requirement is fulfilled by a given pair of reactants, although the failure to meet this requirement in some cases is obvious. Thus, conversion of 1,3-propanediol to the monophosphate, where *three* groups are interposed between the phosphate binding group and the hydroxyl nucleophile, produces a compound that is too short to act as an efficient acceptor, and the rate of PO_3 transfer to the hydroxyl group of $HO(CH_2)_3OP$ is less than 0.002 of that observed in a separate reaction with 0.5 M $HO(CH_2)_3OH$ + HP.[7] Even with *four* groups in the chemical bridge, as are present in the normal substrate, the observed nucleophile-binding effect can be suboptimal. Thus conversion of glycerol to erythritol-4-P, by substitution of $-CH_2OPO_3^{2-}$ for the terminal hydrogen, produces only an eightfold increase in rate based on the same type of comparison as above. However, when C_3 alcohols that contain *no other functional groups,* 1-propanol, allyl alcohol, and propargyl alcohol, are modified by substituting $-CH_2OPO_3^{2-}$ at the terminal carbon, rate increases of 240-, 145-, and 90-fold, respectively, are produced relative to the original 1 M C_3 alcohol in the presence of bound phosphite. As might be expected from the structure of the normal substrate, when five or six groups are used in the bridge, as in $HO(CH_2)_5OP$ or in sorbitol-6-P, marked decreases in reaction rate are observed relative to $HO(CH_2)_4OP$ (0.06- and 0.0006-fold, respectively).

The above rate effects, which can be considered as "intramolecular effects", are quite small, indeed, in comparison with the maximum feasible intramolecular effect of about 10^9-fold (Page and Jencks, 1971). However, the similarity in the size of the nucleophile-binding effect that was observed with the above three C_4-diol monophosphates, in spite of their substantially different stereochemical requirements, plus the rate ratio of only 3.1/1 for the trans and cis isomers of 2-butene-1,4-diol-P, suggests that the structure of the chemical bridge is not critically important in these comparisons and hence that the above values may approach the value for the "ideal flexible bridge" in the present system, although problems due to the possibility of nonproductive binding, or even the lack of binding of the hydroxyl end of a diol monophosphate when its PO_3 group is bound, cannot be evaluated directly.[8] Hence, the largest observed ratio, viz., that for butanediol-P vs. 1-propanol plus phosphite, about 250-fold, will be used as the nucleophile-binding factor (however, see below).

The $(CHOH)_3$-Bridging Factor. The final step in Scheme I (step d) involves conversion of the *bound* diol monophosphate into a glucose-1-P by addition of a second chemical bridge: one containing the $(CHOH)_3$ portion of the glucose ring, i.e., the 2-, 3-, and 4-hydroxymethylene groups. According to Scheme I, the $(CHOH)_3$-bridging effect is equal to the ratio of the overall substrate-induced rate effect, 3×10^{10}-fold, and the product of the increased transfer efficiency in steps a, b, and c, which has been estimated, above, as about 2.5×10^7-fold. However, the validity of this ratio, about 10^3-fold, depends on the validity of the factors for each of the previous steps. Hence, two other schemes for analyzing the substrate-induced rate effect are considered below. In these schemes, the $(CHOH)_3$ bridge is introduced earlier in the sequence in an attempt to make the corresponding rate more directly accessible, experimentally. However, because of the manner in which it is identified, a completely independent evaluation of the $(CHOH)_3$-bridging effect is not feasible.

The Xylose-1-P Reaction. When the 6-hydroxymethyl group of glucose-1-P is replaced by a hydrogen, the xylose-1-P thus produced can no longer function as a PO_3 acceptor. However a water molecule, which apparently occupies the vacant 6-position in the enzyme·xylose-1-P complex, is able to act as a relatively efficient PO_3 acceptor in place of the missing hydroxymethyl group. This reaction appears to be analogous to the increased water reaction of phosphoglycerate mutase with phosphoglycolate (Rose, 1970) and of hexokinase with D-lyxose and D-xylose (cf. Fromm and Rudolph, 1971). Thus, in the hexokinase reaction, 6-deoxyglucose markedly inhibits the water reaction, and in the present case, 6-deoxyglucose-1-P elicits a very much slower water reaction than xylose-1-P (see Table I). However, in the case of phosphoglucomutase, an increase of some 2×10^5-fold in the rate of the water reaction is produced by xylose-1-P[9]—as opposed to 5-fold with xylose and 30-fold with lyxose in the hexokinase system.

According to Scheme II, the 3×10^{10}-fold substrate-induced rate effect is equal to the product of the xylose-1-P factor, 2×10^5-fold (step e), which includes the PO_4- and $(CHOH)_3$-

[7] The reduced PO_3-transfer rate to the C_3-diol-P, relative to the diol plus phosphite, is not caused by inefficient activation of the transfer process by the PO_3 group of the diol-P since $HO(CH_2)_3OP$ produces a water reaction that is some 5000-fold faster than the unactivated water reaction, viz., is some 8-fold faster than that produced by bound phosphite.

[8] The unimportance or marginal importance of (1) nonproductive binding or (2) the mobility of the terminal hydroxyl group of the bound C_4-diol monophosphates is *suggested,* although certainly not proved, by (a) the similarity of the values for the nucleophile-binding factor obtained with the unsubstituted C_4-diol monophosphates (see above), (b) the fact that bound $HO(CH_2)_4OP$ prevents PO_3 transfer to both water and 1 M methanol (although bound $HO(CH_2)_3OP$ does not; see Table I), and (c) the observation that the equilibrium constant for PO_3 transfer to the bound $HO(CH_2)_4OP$ is similar to that for the analogous step involving bound glucose-1-P (Ray and Long, 1976a).

[9] In the presence of xylose-1-P, phosphoglucomutase becomes an inefficient but rather selective glucose bisphosphate phosphatase ($V_{max} \simeq$ 6 nmol min^{-1} mg^{-1}; $K_m \ll 1$ μM at pH 7.5 and 30 °C), and was used in this manner by Ray and Long (1976a). However, when xylose-1-P binds to the complex of dephospho-enzyme and inorganic phosphate, its 1-phosphate group is slowly transferred to the enzyme with the liberation of xylose, so that phosphoglucomutase also can act as an inefficient xylose-1-P phosphatase. The latter reaction can become appreciable at concentrations of inorganic phosphate approaching 0.1 mM. (D. S. Goodin, M.S. Thesis, Purdue University, 1974).

SCHEME II. A Sequence by Means of Which the Water Reaction Can Be Related to the Glucose-1-P Reaction (Cf. Scheme I).

$$[E_p + H_2O] \xrightarrow{e} [E_p \cdot \text{Xyl-1-P} + H_2O] \xrightarrow{f} [E_p \cdot \text{Glc-1-P}]$$

bridging factors of Scheme I, and the factor for step f, which in turn is equal to the product of the C-OH/H-OH and the nucleophile binding factors from Scheme I. The last two factors appear together in Scheme II since bound xyl[-P prevents an observable methanol reaction (see Results e- sumably because of the larger steric requirements [e methanol reaction vis-à-vis the water reaction. If the)4 factor is taken as 1000-fold, as before, the $(CHOH)_3$-bridging factor is 200-fold, which is somewhat smaller than the value of 1000-fold that was suggested by the above analysis of Scheme I. However, it seems more reasonable to use the value of 200-fold for the $(CHOH)_3$-bridging factor because of the more nearly independent nature of this estimate. Since the factor for step e is somewhat smaller than expected from the analysis of Scheme I, the value for the factor in step f must be somewhat larger than expected. However the agreement between the product of the C-OH/H-OH and the nucleophile binding factors from Scheme I, 0.25×10^5, and the value of 1.5×10^5-fold for step f is reasonable.

The Xylose plus Phosphite Reaction. Scheme III shows a three-step sequence in which the substrate-induced rate effect

SCHEME III. A Sequence of Changes by Means of Which the Water Reaction Can Be Related to the Glucose-6-P Reaction (Cf. Scheme I).

$$[E_p + H_2O] \xrightarrow{g} [E_p \cdot \text{Xyl}] \xrightarrow{h} [E_p \cdot \text{Xyl-HP}] \xrightarrow{j} [E_p \cdot \text{Glc-6-P}]$$

involves the ratio of the PO_3-transfer rates to water and to the 1-position of glucose-6-P, about 10^{10}-fold. However, it is difficult to relate the factors for steps g to j in this scheme to the factors for steps a to d in Scheme I, for a variety of reasons, only one of which is noted below, and although an attempt to formulate a relationship between these sets of factors ultimately may be desirable, additional information will be required. Thus, since the factor for step h that apparently is analogous to the PO_4 factor of Scheme I is 30 000 instead of 1000-fold as in step a of Scheme I, the PO_4 effect may be more important in PO_3 transfer to the 1-hydroxyl than to the 6-hydroxyl group of glucose phosphate. Although it might be argued that this difference is simply related to introducing the PO_4 factor later in Scheme III than in Scheme I, the epimeric position of the 2- and 4-hydroxyl groups also is much more important in the more difficult PO_3 transfer to the 1- than the 6-hydroxyl group (cf. Lowry and Passonneau, 1969), and the latter difference is unrelated to schemes for dissecting the substrate-induced rate effect.

The Intrinsic Binding Energy of the PO_3 Group of Glucose 1-Phosphate. In the previous sections, only effects on the *rate* of PO_3 transfer induced by different parts of the normal substrate were considered. However, Jencks and Page (1972) make a strong case for formulating arguments in terms of intrinsic binding energies when analyzing substrate-induced rate effects. Since true values for intrinsic binding energies are difficult to estimate, values of $-RT \ln k_{uni}/K_d$ were used instead, where k_{uni} is the unimolecular rate constant for PO_3 transfer and K_d is the dissociation constant for the acceptor or activator in question. Briefly, the rationale for such an approach is that part of the intrinsic binding energy of activators or of acceptors that cause an increase in the PO_3-transfer rate must be expended in producing energetically unfavorable changes in the system. Although this approach still does not provide reliable estimates of intrinsic binding energy, only *differences* in the energies thus obtained are considered, and these *differences* may well approximate *differences* in intrinsic binding energy for structurally similar acceptors or activators. However, a precise interpretation is less obvious for dissimilar acceptors and differences in "observable binding energy" will be implied in all cases noted below. In comparisons involving the water reaction as the reference, $(k_{uni}/K_d)_{H_2O}$ is taken as equal to the measured rate constant k_{obsd} (see below). This procedure gives the Gibbs energy change associated with the following hypothetical process, involving acceptor, A-OH,

$$\text{A-OH} + \left[\begin{array}{c} \quad H \\ \quad \vdots \\ E \cdots P \cdots O\text{—}H \end{array}\right]^{\ddagger} \longrightarrow \left[\begin{array}{c} \quad H \\ \quad \vdots \\ E \cdots P \cdots O\text{-A} \end{array}\right]^{\ddagger} + H_2O$$

in which a water proton in the transition state for the water reaction is replaced by the A group of the A-OH acceptor, and whatever additional changes are required to produce the transition-state leading to A-OP also are made (cf. Scheme I, Ray and Long, 1976a). When glucose-1-P is the acceptor, this energy difference is equal to some −21.5 kcal/mol and is some −19.7 kcal/mol for glucose-6-P. (The steady-state value of k_{cat} is used as a measure of k_{uni} for PO_3 transfer to glucose phosphates, although k_{cat} may be somewhat smaller than k_{uni}.) Although these energy differences may arise entirely from the intrsic binding energy of the glucose phosphates, it is difficult to account for all of these differences in this manner and the actual binding energy of the glucose phosphates may not be quite as negative as these values suggest. Thus, nothing is known about the status of water at the active site of the enzyme, and taking k_{uni}/K_d for the water reaction as equal to k_{obsd} is equivalent to setting K_d for water equal to 1 M (if the unit convention is to be maintained), and this seems [illegible]stified.

However, about −8 kcal/mol of the observable difference in binding energy, calculated from the glucose-1-P and water reactions, can be accounted for by dianionic PO_3 or PO_4 groups, as is estimated from the measured binding constant for inorganic phosphite, 500 M^{-1} (Ray and Long, 1976a), and the PO_4 factor from Scheme I, 1000-fold. Moreover, an additional −1.3 kcal/mol is accounted for if the binding of phosphite is evaluated in the presence of bound xylose (binding constant, 200 M^{-1} (Ray and Long, 1976a) and increased rate of PO_3 transfer, 30 000 (see above)), so that the overall energy difference under these conditions is nearly half of the 19.7 kcal/mol energy difference calculated for water and glucose-6-P.

Presumably Coulombic effects provide most of the overall binding energy of the PO_3 moiety but the increase in the PO_3-transfer rate produced by the binding of this group is only an indirect result of Coulombic effects. Thus, the binding energy of glucose 6-sulfate, which has one less negative charge and thus should serve as a reasonable model for monoanionic glucose-6-P, is about 4.4 kcal/mol less negative than that of the dianionic phosphate (it binds some 0.0015 as well and reacts about 0.4 as fast; see Results). Hence, the overall binding energy for neutral glucose-6-P should be about 8.8 kcal/mol less negative than that for the dianionic species, and this difference thus accounts for most of the overall effect produced by the binding of inorganic phosphite in the presence of bound xylose, −9.3 kcal/mol (see above). Thus, the rate effects produced by bound phosphite apparently are the direct result of its size and shape, while its Coulombic charge provides only the driving

force for utilizing these properties. Such an interpretation also is in accord with the observation that the binding of xylose and phosphite elicits nearly the same rate of PO_3 transfer as bound glucose-6-P, and would produce the same overall effect if only a small chelate effect were invoked (cf. Ray and Long, 1976a). Note also that, although binding phenomena and catalytic events frequently are inseparable, the markedly reduced binding of glucose 6-sulfate, relative to glucose-6-P, scarcely affects the ability of the 1-hydroxyl group of the sugar ring to act as a PO_3 acceptor subsequent to the binding step.

An overall binding energy of −9.3 kcal/mol is more negative than one might expect for ion-pair formation in aqueous solution. (The association of SO_4^{2-}, for example, even with tripositive metal ions at $\mu \rightarrow 0$, does not involve energy changes significantly more negative than −5 kcal/mol (Sillen and Martell, 1964).) However, the *measured* binding energy for inorganic phosphate in at least one enzymic system, alkaline phosphatase (Applebury et al., 1970), is nearly −10 kcal/mol. In fact, a detailed rationale of phosphate binding affinity both in the present system and in the alkaline phosphatase system ultimately may require consideration of a binding site with a somewhat elevated chemical potential, viz., a site that is poorly "solvated" by water and more efficiently solvated by bound phosphate. (The central cavity in cyclic dextrans is an example of a (polar) site that is poorly solvated by water (Thoma and Stewart, 1965); although its structure is not conducive to binding of phosphate, the same principle could be used to construct a "high energy" phosphate-binding site.)

General Conclusions

One of the most interesting aspects of this study is the observation that a substantial fraction of the very large substrate-induced rate effect in the phosphoglucomutase system does not depend on covalent linkages between different parts of the substrate when these parts are bound at the active site of the enzyme. This is illustrated by the increased rate of PO_3 transfer to water induced by simple phosphate esters and xylose-1-P, and by the near optimal efficiency of xylose as a PO_3 acceptor in the presence of bound phosphite. Thus, simple phosphate esters, which must bind several bond lengths away from the site of the PO_3-transfer process, produce an increase in the transfer rate to water of up to 1000-fold. It seems quite improbable that the binding of such esters exerts any direct effect on the attacking water molecule because of the physical separation between the two, and their binding also does not significantly alter the thermodynamic stability of the enzymic phosphate group. Hence, the primary effect of the activating PO_4 group probably is on the superstructure of the enzyme rather than on the reactant groups.

The binding of xylose-1-P, which also includes the effect of the $(CHOH)_3$ group of the sugar ring (see below), also fails to produce an increase in the chemical potential of the enzymic phosphate. (Its chemical potential actually decreases by about 1 kcal/mol (Ray and Long, 1976a).) This failure (which is a consequence of the not very disparate affinity of xylose-1-P for the phospho and dephospho forms of the enzyme; cf. Ray and Long, 1976a) suggests, but does not prove, that the phosphate group and sugar ring of this activator also exert their primary effect on the superstructure of the enzyme, as in the case of simple phosphate esters, and not on the enzymic phosphate group or on the water acceptor.

It is not clear whether the 3×10^4-fold rate increase provided by the binding of inorganic phosphite in the presence of bound xylose should be considered as a special PO_4 effect for PO_3 transfer to the moderately shielded, secondary C-1 hydroxyl group or whether the binding of phosphite also acts to compensate for suboptimal positioning of bound xylose (see previous sections). However, the reaction does show that near-maximal rate reffects can be produced by what appear to be largely independent binding interactions. Thus, inorganic phosphite binds nearly as tenaciously in the absence of xylose as in its presence and, conversely, xylose binds nearly as well in the absence of phosphite as in its presence;[10] in addition, the overall binding effect for inorganic phosphite in the presence of xylose approximates the overall effect of a $-CH_2OP$ group covalently attached to xylose (Ray and Long, 1976a).

Although the precise meaning of these observations is unclear from the standpoint of the catalytic mechanism, it is obvious that binding interactions can produce large rate effects at a reaction site some distance from the enzyme–activator contact, without depending on covalent bonds between the activator and acceptor moieties to transmit the effect of activator binding to the acceptor group. In one sense, the above examples might be considered as a type of allosteric activation (Monod et al., 1965), although demonstrated allosteric effects as large as 2×10^5-fold are rare. Moreover, it is not clear whether a substantial conformational change accompanies the above binding phenomena so that an "induced fit" type of activation (Koshland, 1963) is implied, or whether an overall tightening of enzyme–enzyme and enzyme–substrate interactions, as emphasized by Page and Jencks (1971), is the important aspect of the PO_4-binding process. However, the present suggestion that it is the size and shape of the activating PO_4 group that is important in catalysis—not its charge—seems more in keeping with the former possibility.

Other differences in the water reaction and the glucose-1-P reaction are easier to relate to the catalytic mechanism. Thus the effect of pK_a on acceptor reactivity indicates that general-base catalysis is important in the PO_3 transfer to simple alcohols and, although the Bronsted plot in Figure 1 shows extensive scatter, a substantial degree of proton transfer from the acceptor hydroxyl group to a base at the catalytic site of the enzyme is suggested.[11] However the majority of the 40-fold greater efficiency for 1 M methanol relative to 55 M water, which accounts for the majority of the suggested C–OH/H–OH factor of 100-fold, cannot be rationalized in terms of a pK_a difference. (On a molar basis the rate difference would be about 2000-fold while the K_a difference only is about 16-fold.) Although increased binding interactions of a methyl group with the enzyme might account for this difference, the possibility that successful attack on the enzymic PO_3 group requires a greater desolvation of a water molecule than of a methanol molecule seems more attractive. Such a difference

[10] The subsequent binding of inorganic phosphate in the case of xylose or of inorganic phosphite in the case of glucose produces much smaller rate increases than binding of phosphite in the presence of bound xylose (see Results). This is not surprising from an examination of models of a binding site into which glucose-6-P would fit snugly. Such models suggest that significant distortion of a glucose-6-P binding site would be required for simultaneous binding of glucose plus phosphite or xylose plus phosphate, but no distortion would be required for binding of xylose plus phosphite and the relative PO_3-transfer rates, 0.9, 1.1, and 100, respectively, at 1 M sugar and 25 mM (nearly saturating) phosphate or phosphite support this expectation.

[11] In their study of the reaction of oxy anions with *p*-nitrophenyl acetate, Jencks and Gilchrist (1962) also observed substantial scatter in a Bronsted plot and noted that the anions of methanol and propargyl alcohol are more reactive, and those of chloroethanol and ethylene glycol methyl ether were less reactive than expected in terms of the pK_a of the corresponding alcohols. This pattern also is seen in Figure 1 and may be related to differences in the required desolvation of the attacking hydroxyl group or oxy anion.

might occur if the approach of solvent or solute molecules to the enzymic phosphate was markedly restricted in the free phospho-enzyme.

A general interpretation of the nucleophile-binding factor also is straightforward, although, as noted above, the observed value is quite small in comparison with the maximum possible entropic intramolecular effect of some 10^9-fold (Page and Jencks, 1971). However, since translational and rotational immobilization of the reacting groups (Page and Jencks, 1971) probably is responsible for most of the nucleophile-binding factor, a value of 250-fold for this factor is not unreasonable. Thus, the immobilization of water at 30 °C by "freezing" would require a decrease only of about 6 eu/mol (cf. Daniels, 1948), and an immobilization of reacting groups equivalent to this would favor an intramolecular process over the corresponding intermolecular process only by about 20-fold (if the immobilization occurred prior to and was not included in the rate determining step of the former process, but occurred during the slow step of the latter process). Even the complete elimination of the motional entropy of water (which is about 16 eu at 25 °C; cf. Daniels, 1948) would produce a rate factor only of about 5000-fold, and it is unlikely that binding interactions could immobilize the hydroxyl group of a diol monophosphate (relative to the enzymic phosphate), even one that had an "ideal" flexible bridge, to anything like this extent. (At room temperature, only crystalline *solids* with *covalent* lattices such as diamonds (S = 0.9 eu/mol at 25 °C; Daniels, 1948) have essentially no motional entropy.) Hence, a value of 250-fold for the nucleophile-binding effect, as defined in Scheme I, is not unreasonable, although the possibility that a somewhat larger factor should be associated with the intramolecular effect for glucose-1-P can not be ruled out (see below).

A relative small nucleophile-binding factor also is in accord with the observation that only a small chelate effect is associated with the attachment of a $-CH_2OP$ group to the 5-position of xylose to give glucose-6-P (Ray and Long, 1976a). Thus, extensive group immobilization is the basis for both large chelate effects and large nonenthalpic intramolecular rate enhancements; hence, large values of the rate factor for nucleophile binding are not expected in the absence of a large chelate effect for the nucleophile binding step.

If, in the xylose-1-P induced PO_3 transfer to water, the binding of the sugar ring immobilizes and enthalpically destabilizes a water molecule in the position normally occupied by the 6-hydroxyl group of glucose-1-P, it might be more reasonable, from a mechanistic standpoint, to drop the $(CHOH)_3$-bridging factor and to increase the nucleophile-binding factor by a commensurate amount. In such a case, the augmented nucleophile-binding factor would be by far the largest of the remaining three factors. However, the following considerations do not support making such a combination. (a) The pronounced glucose phosphate induced spectral changes in the enzyme, which may be related to induced structural changes (Yankeelov and Koshland, 1965), are markedly altered by changes in the $(CHOH)_3$ bridge (W. J. Ray, Jr., unpublished results). (b) In addition, the structural changes in the enzyme that *accompany* the actual PO_3 transfer during interconversion of E_P·Glc-P and E_D·Glc-P_2 (Ray and Long, 1976b) and whose mechanistic significance is not understood appear to be related to the sugar ring. On the other hand, the efficiency of PO_3 transfer is surprisingly insensitive to some changes in the $(CHOH)_3$ portion of the sugar ring. Thus, the decrease in catalytic efficiency that accompanies epimerization of the 2-hydroxyl group of glucose-1-P (as in mannose-1-P) is only 15-fold (Lowry and Passonneau, 1969) while eliminating the 2- and 3-hydroxyl groups and contracting the ring (as in 2-deoxyribose-1-P) produces only a 30-fold decrease, i.e., PO_3 transfer to the latter acceptor still is some 10^9-fold faster than to water (W. J. Ray, Jr., unpublished results).

The product of the four factors that were identified in terms of Scheme I (1000-fold for the PO_4 factor, 100-fold for the C-OH/H-OH factor, 250-fold for the nucleophile-binding factor and 200-fold for the $(CHOH)_3$-bridging factor) is about 5×10^9-fold and thus is close to the overall substrate-induced rate effect, 3×10^{10}-fold. The question as to whether this relatively close agreement supports the present attempt to dissect the substrate-induced rate effect into mechanistically distinct effects, or is only an operational necessity, is a difficult one. However, we feel that, in view of the variety of reactions that were explored in attempting to evaluate the factors that were chosen, a cautiously affirmation reply is warranted. To say the least, for a given four-step sequence analogous to Scheme I, *small* factors for steps a, b, and c might well be expected, instead, so that an enormous factor would remain for step d. In such a case the latter factor could more appropriately and less informatively be referred to as a "putting-it-all-together" factor. In fact, a huge and abrupt change for the final step in analogous sequences for other enzymes may be the rule, as opposed to the graded, factorial series of effects found for the phosphoglucomutase system.

Finally, the apparent success encountered in rationalizing the overall substrate-induced rate effect in terms of the contributions of four different parts of the glucose phosphate molecule supports the position taken in an accompanying paper that the majority of the substrate-induced rate effect cannot be rationalized in terms of any single mechanism for using substrate-binding energy to produce an increased PO_3-transfer rate (Ray and Long, 1976a) and thus must represent a composite of several more modest effects.

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