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Anomalous Mutarotation of Glucose 6-Phosphate. An Example of Intramolecular Catalysis*

J. M. Bailey, P. H. Fishman, and P. G. Pentchev

ABSTRACT: Glucose 6-phosphate is metabolized by enzymes for which distinct anomeric specificities have recently been demonstrated. The mutarotation of glucose 6-phosphate was measured by utilizing the reduced triphosphopyridine nucleotide coupled rate of oxidation with glucose 6-phosphate dehydrogenase. This enzyme is specific for the β anomer. The mutarotation was 240 times faster than that of α-D-glucose measured polarimetrically under the same conditions. It was found that the higher rate was not caused by increased ring strain, since the activation energy for the mutarotation of glucose 6-phosphate (21.8 kcal/mole) was essentially the same as for D-glucose (22.2 kcal/mole). The mutarotation of glucose and a number of other sugars was shown to be accelerated by inorganic phosphate. The pH and concentration dependence of this reaction were determined. The theoretical mutarotation rate of glucose 6-phosphate, based upon a model of intramolecular catalysis, was calculated from these results to be 0.97 min^{-1}. This was in excellent agreement with the experimentally observed value of 1.1 min^{-1} at 10°. In addition, glucose 6-phosphate accelerated the mutarotation of free glucose in the expected manner. It is concluded that the explanation for the anomalous mutarotation of glucose 6-phosphate lies in an intramolecular catalysis of the mutarotation reaction at the anomeric OH by the phosphate group at C-6 of the molecule.

The compound glucose 6-phosphate (G-6-P) is of central importance in glucose metabolism. The anomeric specificities of the enzymes metabolizing glucose and glucose 6-phosphate have recently been defined [Salas *et al.* (1965); Bailey *et al.* (1968)]. Enzymes involved in the phosphorylation of glucose (hexokinase, glucokinase, and pyrophosphate phosphotransferase) display no preference for either the α or β anomer of D-glucose. The anomeric configuration of the G-6-P produced is thus the same as that of the glucose used as substrate. Similarly, it has been shown that the enzymes dephosphorylating glucose 6-phosphate produce free glucose which has the same anomeric composition as the substrate [Bailey *et al.* (1968)]. It has been shown by Salas *et al.* (1965), however, that the enzymes involved in the further metabolism of G-6-P do display marked preferences for the particular anomer used as substrate. Thus the enzyme glucose 6-phosphate dehydrogenase which channels glucose metabolism into the pentose phosphate pathway is completely specific for the β anomer. As described below, we have used this fact to measure the anomeric composition and mutarotation rate of glucose 6-phosphate under different experimental conditions.

The main glycolytic pathway for glucose metabolism is initiated *via* the α anomer of G-6-P by the enzyme phosphoglucose isomerase. This enzyme is thought to act on the open-chain form of glucose 6-phosphate as an intermediate (Salas *et al.* (1965)]. This was shown to be derived from the α anomer under the catalytic action of phoshoglucose isomerase functioning as a "mutarotase." The enzyme mutarotase itself, which occurs in many tissues, particularly kidney, liver, and intestine, is specific for the free sugar and has no action on either anomer of glucose 6-phosphate [Bailey *et al.* (1968)].

The enzyme phosphoglucomutase is also specific for the α

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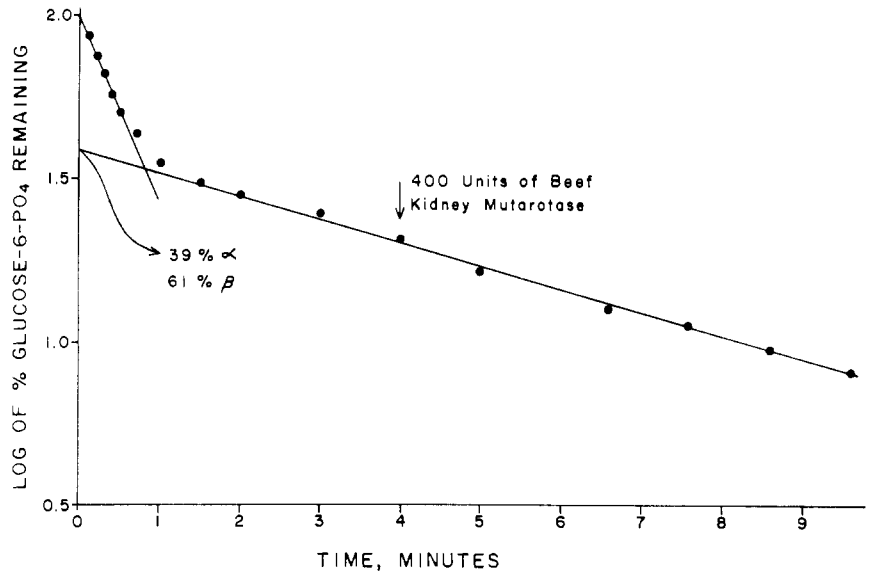

FIGURE 1: Measurement of mutarotation rate of G-6-P by use of glucose 6-phosphate dehydrogenase. G-6-P (0.12 μ-moles) was incubated in 2 ml of Tris buffer (32 mM, pH 7.8) containing 1.0 μmole of TPN, 2.6 μmoles of $MgCl_2$, and 5 units of glucose 6-phosphate dehydrogenase. Reaction was initiated by addition of the enzyme and TPN reduction was monitored at 340 mμ with the Beckman Model DBG spectrophotometer equipped with a refrigerated cell compartment and coupled to a high speed recorder. Reaction temperature was 0°. By plotting the logarithm of the percentage of sugar remaining, against time, the mutarotation rate of α-glucose 6-phosphate to the oxidizable β anomer was obtained from the slope of the line representing the second part of the biphasic rate curve. The percentage of β anomer in the equilibrium mixture at zero time is obtained from the intercept of this line with the ordinate at zero time. The failure of 400 units of mutarotase, added at 4 min, to increase the mutarotation rate demonstrates that G-6-P is not a substrate for this enzyme.

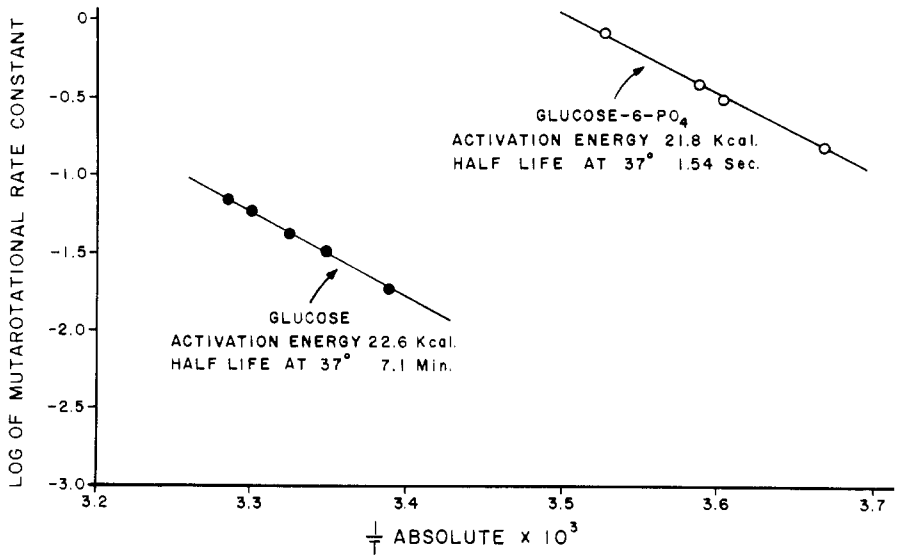

FIGURE 2: The mutarotation rate of G-6-P was measured at different temperatures in the range of 0 to 10.5° as described in the footnote to Figure 1. The mutarotation of 0.3% solutions of α-D-glucose was measured over the range 15–35° by the automated polarimetric assay described in the Methods section.

anomer of G-6-P, and the glucose 1-phosphate produced is entirely in the α configuration. Conversely, therefore, the glucose 6-phosphate produced as the result of glycogen breakdown will be entirely of the α configuration.

It was demonstrated by Salas *et al.* (1965) that the potential disruptions in the partitioning of glucose 6-phosphate among the various metabolic pathways is overcome by a very fortunate circumstance in that the spontaneous mutarotation rate of G-6-P is over 100 times greater than that of free glucose. In confirming Sols' observation, it was shown that whereas the half-time for the interconversion of the anomers of free glucose under physiological conditions is about 7 min, the half-time for the interconversion of α- and β-glucose 6-phosphate is only 1.5 sec [Bailey *et al.* (1968)].

It seemed reasonable to assume that the increase in mutarotation rate of G-6-P was caused by increased ring strain brought about by the bulky phosphate group at C-6. This would be expected therefore to lower the activation energy for the reaction. It was found however that although the rate is some 240 times faster, the activation energy for the mutarotation of glucose 6-phosphate (21.8 kcal) is essentially the same as that of free glucose (22.2 kcal).

It is shown in this paper that the probable explanation for this anomalous behavior lies in an intramolecular mechanism of catalysis of the mutarotation reaction at C-1 by the phosphate group at C-6 acting in a dual acid–base capacity.

Materials and Methods

Na_2G-6-P, D-glucose, D-xylose, D-fucose, and α-lactose were from Nutritional Biochemicals. 2-Deoxy-D-glucose was the gift of Dr. W. J. Whelan.

Mutarotation rates of the simple sugars were determined using a Bendix type 143A automatic polarimeter coupled to a Beckman 12-in. chart recorder operated in the logarithmic mode. The polarimeter cell (path length 40 mm) was water jacketed and supplied from a refrigerated water bath so that any desired temperature between 0 and 37° was maintained to within ±0.1°. A variable speed Sigma motor pump was used to fill the cell.

For routine assay, a sample (36 mg) of the crystalline α anomer of the test sugar was dissolved in 12 ml of the appropriate test buffer. The solution was rapidly introduced into the polarimeter tube by means of the pump and flow-through assembly to flush out the tube without introduction of air bubbles. The recorder was activated at the same time as the buffer solution was added. The recorder was equipped with logarithmic gears, so that by suitable precalibration of the recorder and polarimeter for the particular sugar used, the first-order rate constants for the mutarotation reaction were obtained directly from the slope of the linear plot on the chart [Bailey *et al.* (1967)].

The mutarotation of glucose 6-phosphate was measured by a coupled reaction utilizing glucose 6-phosphate dehydrogenase. The rate of conversion of β-glucose 6-phosphate into β-phosphogluconate was monitored by following reduction of added TPN at 340 mμ with the Beckman Model DBG spectrophotometer. This was coupled to a 12-in. Beckman recorder operated at a chart speed of 10 in./min. The reactions were carried out at low temperatures (in the range of 0 to 15°) by circulating coolant through the cuvet holder. Condensation of water vapor on the optically plane surface of the cuvets was overcome by spraying lightly with windscreen demister (Firestone stores). Following the initial rapid oxidation of β-glucose 6-phosphate which represents about 60% of the equilibrium mixture, the mutarotation of the remaining α-glucose 6-phosphate becomes the rate-limiting factor. The mutarotational rate constant for G-6-P was derived from the slope of the line obtained by plotting log per cent glucose 6-phosphate remaining *vs.* time, as illustrated in Figure 1.

Results and Discussion

The mutarotation of glucose 6-phosphate is too rapid to be measured by conventional polarimetric methods. Use was made of the specificity of glucose 6-phosphate dehydrogenase for the β anomer. The progress of the reaction, starting with the equilibrium mixture of G-6-P, was followed by monitoring TPN reduction spectrophotometrically in refrigerated cuvets. By use of a high-speed recorder, the biphasic nature of the oxidation was demonstrated (Figure 1). The initial rapid phase of oxidation corresponding to utilization of the β anomer already present in the solution was followed by a slower reaction in which mutarotation of the α anomer to the oxidizable β form was the rate-limiting step. The slope of this second portion gave the first-order mutarotational rate constant directly. The point of intersection of this line with the ordinate gave the anomeric composition of the glucose 6-phosphate. These assays were carried out at a number of different temperatures. The activation energy for the mutarotation reaction was measured by plotting the logarithm of the rate constant against the inverse of the absolute temperature according to the conventional Arrhenius treatment. The absolute mutarotation rate of G-6-P was approximately 240 times that of D-glucose under the same conditions. The activation energy for the reaction (21.8 kcal) however was not significantly different from that of D-glucose measured in dilute EDTA buffer (Figure 2 and Table I).

TABLE I: Relative Activation Energies and Mutarotational Rate Constants for Sugars at pH 7.0.[a]

	Mutarotation Rate (D-glucose = 1)	Activation Energy (kcal/mole)
D-Glucose-6-PO_4	240	21.8
D-Glucose + H_2O	1	22.2
D-Glucose + PO_4	21.3	17.4
D-Galactose + H_2O	1.1	21.4
D-Galactose + PO_4	15.4	17.4
D-Xylose + H_2O	3.3	22.8
D-Xylose + PO_4	25.4	21.8

[a] Mutarotation rates of the various sugars were measured at a series of temperatures in the range 0 to 37° as described in the Methods section, either in water or in 0.12 M phosphate buffer (pH 7.0). The activation energies were derived from conventional Arrhenius plots as illustrated in Figure 2 for glucose and G-6-P.

From *a priori* considerations, it seemed reasonable to assume that the rapid mutarotation of G-6-P compared with glucose was related to increased ring strain due to the presence of the bulky phosphate group at carbon 6. This in turn would lead to an increase in the ease of ring opening to the aldehyde or open-chain form which is the presumed intermediate of the mutarotation reaction [Los *et al.* (1956)]. This increased ring strain however would be expected to be manifested in a change of the activation energy, E, of the reaction. Since no such change in E is observed, and since no obvious compensating factors are evident, it must be concluded that the ring-strain hypothesis is not a valid explanation. A second hypothesis, that of intramolecular catalysis as described below, was therefore considered.

It is well known that inorganic phosphate accelerates the mutarotation of glucose [Los *et al.* (1956); Cantor and Peniston (1940)]. Cantor and Peniston (1940) claimed that the phosphate-accelerated mutarotation did not proceed by the normal mechanism, since no increase in the open-chain form could be detected by polarographic analysis. It was later shown by Los *et al.* (1956) however that the phosphate-accelerated mutarotation does indeed proceed by the normal mechanism involving the open-chain or aldehyde form as an intermediate. The rate-limiting step both in ring opening and ring closing is a base-catalyzed removal of a proton. Thus phosphate ion accelerates the rate of ring opening and ring closure to an approximately equal extent, and there is no significant change in the concentration of the open-chain form with increasing mutarotation rate. It seemed possible therefore that the increased mutarotation rate of G-6-P may have been related to a catalysis of the mutarotation reaction at carbon 1 by the phosphate group at C-6.

In order to test this hypothesis, the mutarotation rate of

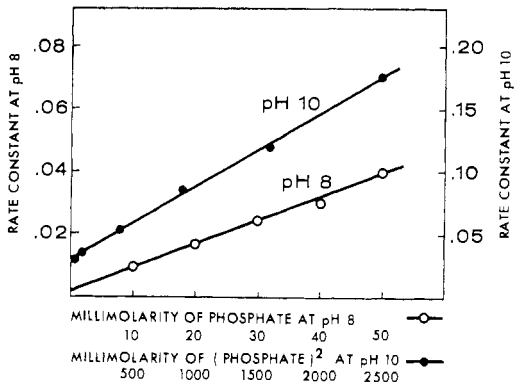

FIGURE 3: Mutarotation rate of 0.3% solutions of α-D-glucose was measured in phosphate buffers of pH 8 and pH 10 over a range of phosphate ion concentrations. At pH 8, the rate was approximately proportional to the first power of the phosphate ion concentration but at pH 10 the rate was more nearly proportional to the square of the phosphate ion concentration. At pH 8 and a temperature of 10° with increasing phosphate ion above the 50 mM range shown here, a point of inflexion was reached and a second linear relationship between the concentration of phosphate and mutarotation rate was attained. This has a slope of approximately half that for the 0 to 50 mM range shown here. This second portion of the curve, *i.e.*, the range 50 to 300 mM phosphate, was used for extrapolation of the rate to the value of 2.2 M phosphate used in the calculations in the Results and Discussion section.

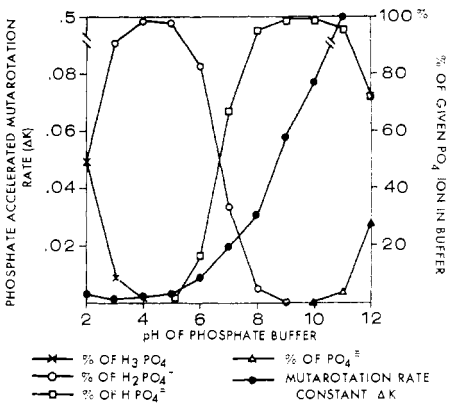

FIGURE 4: The mutarotation rate of 0.3% solutions of α-D-glucose was measured using the semiautomated polarimetric assay system described in the methods section. Reaction was carried out in 50 mM phosphate buffers at pH values of 2, 4, 6, 8, and 10, at a temperature of 20°. The spontaneous rate measured in 1 mM phosphate buffer at each pH value was subtracted to give a measure of the true phosphate-catalyzed portion of the mutarotation reaction at the various pH values. The proportions of the various ionic forms of phosphate were calculated from the Henderson–Hasselbalch equation by conventional procedures.

free glucose was measured in phosphate buffers of increasing concentration. It was found that at pH 8, the pH at which the measurements on glucose 6-phosphate had been made, there was a linear relationship between the phosphate-accelerated mutarotational rate constant and phosphate ion concentration (Figure 3).

A scale model of the glucose 6-phosphate molecule was constructed. It was found that the freely rotating esterified primary hydroxyl group at C-6 allows ready contact between the oxygen of the phosphate group and the anomeric hydroxyl group of the glucose molecule, whether this is in the α or β configuration.

The specific reaction rate of a chemical reaction is related to the activation energy E by the equation, $k = PZe^{-E/RT}$, where the term $e^{-E/RT}$ is the fraction of the molecules having sufficient energy to react, Z represents the frequency factor, *i.e.*, the total frequency of encounters between two reactant molecules, irrespectively of whether they possess sufficient energy or not, and P is the probability that a collision between activated molecules actually will lead to a reaction. The collision factor Z would be expected to be increased by the close proximity of the phosphate group at carbon 6 to the anomeric hydroxyl group at C-1. It is likely also that the probability factor P, which depends upon such factors as the relative steric configurations of the colliding reactants, would also be different.

Our calculations however show that the major portion of the rate increase in the mutarotation of G-6-P can be attributed to the collision factor Z alone. These conclusions were derived from the following considerations. From the model of the glucose 6-phosphate molecule, the average distance from the phosphorus atom at carbon 6 to the anomeric hydroxyl group at C-1 was measured and found to be 7.7 Å. That concentration of phosphate buffer was now calculated in which the average distance between phosphate groups and the C-1 of dissolved molecules of free glucose was the same as that between the phosphate group and C-1 of glucose 6-phosphate. From the properties of the cubic lattice, it can be shown that the average required distance D between phosphate groups is given by $0.75D^2 = 7.7^2$ from which $D = 8.9$ Å.

The molarity of phosphate buffer required to give this average distance is the total number of phosphate atoms per liter divided by Avagadro's number, thus, $(10/(8.9 \times 10^{-8}))^3 \cdot (1/6.023 \times 10^{23})$, which gives 2.22 M as the required concentration.

The expected mutarotational rate constant for glucose at 10° in 2.2 M phosphate buffer was obtained by extrapolation of the measured relationship between mutarotation rate and phosphate concentration as described in the legend to Figure 3. This gave a calculated value for the mutarotational rate constant for G-6-P of 0.97 min^{-1}. The actual mutarotation rate at this temperature obtained from the rate observed in the temperature range 0 to 10.5° shown in Figure 2 is 1.1 min^{-1}.

This excellent agreement between the observed and calculated mutarotation rate for G-6-P, which is based upon the frequency of collision between the phosphate group and C-1, lends strong support to the proposed model of intramolecular catalysis. It also demonstrates that the probability factor P is essentially the same for the phosphate group in covalent linkage at C-6 as it is for free phosphate ions in solution.

The nature of the catalytic function of the phosphate group was examined further. By measuring the mutarotation rate in phosphate buffers of different pH and by substracting the spontaneous mutarotation rate at each pH, the phosphate-accelerated portion of the mutarotation was derived (Figure 3). This in turn was related to the dominant phosphate ion present at each value (Figure 4 and Table II). It was found that the triply charged phosphate ion was the most efficient catalyst, followed in order by the doubly charged ion and un-ionized phosphoric acid. A quantity known as the catalytic coefficient

TABLE II: Catalytic Coefficients for Ion-Catalyzed Mutarotation of Glucose.[a]

	Catalytic Coefficient (moles of α-glucose converted per mole of ion per min at 25°)				
Phosphate					
Ionic species	PO_4^{3-}	HPO_4^{2-}	$H_2PO_4^-$	H_3PO_4	
	0.59	0.071	0.003	0.006	
Arsenate					
Ionic species	AsO_4^{3-}	$HAsO_3^{2-}$	$H_2AsO_4^-$	H_3AsO_4	
	1.34	0.075	0.0015	0.003	
Pyrophosphate					
Ionic species	$P_2O_7^{4-}$	$HP_2O_7^{3-}$	$H_2P_2O_7^{2-}$	$H_3P_2O_7^-$	$H_4P_2O_7$
	0.14	0.030	0.006	0.000	0.012

[a] The mutarotation rate of 0.3% solution of α-D-glucose was measured in phosphate, arsenate, or pyrophosphate buffers of different pH values. For the lower pH values in the range 2 to 8 the reaction was carried out at 15°. For pH values above 8, where the rates of mutarotation became too rapid, the measurements were made at 10° and corrected back to the corresponding rate at 25°. For each determination the mutarotation rate in 1 mM buffer was subtracted from the rate in 50 mM buffer of the same pH value. In this way the specific ion associated acceleration of mutarotation was obtained independently of direct pH effect. The ionic composition for each pH value was calculated from the Henderson–Hasselbalch equation as illustrated by the results in Figure 4. The catalytic coefficient for each specific ion in solution is defined as the moles of α-glucose converted into β- per mole of ion per minute. This was obtained from the calculated ionic composition at each pH value using a set of simultaneous equations describing the rate at each pH in terms of the ionic composition and the catalytic coefficients.

was calculated. This was defined as the moles of α-glucose converted into β-glucose per mole of ion per minute. On this basis the catalytic coefficients for PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, and H_3PO_4 were 0.59, 0.071, 0.003, and 0.006, respectively.

The concentration dependence of the phosphate-accelerated mutarotation was also measured at a number of pH values. It was found that at pH values 2, 4, and 8 the rate was essentially directly proportional to the concentration of phosphate (Figure 3). At pH 10, however, the rate was approximately proportional to the square of the phosphate ion concentration.

These findings support the idea that the phosphate ion functions as a conventional basic reagent extracting a proton from the anomeric hydroxyl group and thus accelerating the ring-opening reaction. However, the dual acid–base catalyzed nature of mutarotation first suggested by Lowry and Richards (1925) is also demonstrated by the greater efficiency of H_3PO_4 than $H_2PO_4^-$. The dependence of the rate at pH 10 upon the square of the phosphate ion concentration is probably related to this also. At pH 10, the PO_4^{3-} ion concentration is small, only about 1%, but a significant part of the rate is attributable to this ion since its catalytic

TABLE III: Relative Acceleration of Mutarotation of Sugars by Phosphate Ion at pH 7.0.[a]

Sugar	Mutarotational Rate Constant		Relative Acceleration
	In Water	In PO_4 Buffer	
D-Glucose	0.014	0.298	21.3
D-Xylose	0.046	0.355	7.9
D-Galactose	0.015	0.216	14.4
D-Fucose	0.051	0.530	10.4
D-Fructose	0.193	>1.6	>8
2-Deoxy-D-glucose	0.193	>1.6	>10
α-Lactose	0.008	0.174	20.7

[a] Mutarotation rates of the various sugars were measured at 25° either in water or in 0.12 M phosphate buffer, pH 6.0. Mutarotation of all sugars tested was accelerated by phosphate to approximately the same degree. Since D-fucose and D-xylose have no free hydroxyl group at carbon 6, this is fairly good evidence that the phosphate-accelerated mutarotation of hexoses does not involve complex formation between carbon 6 and carbon 1 of the sugar ring.

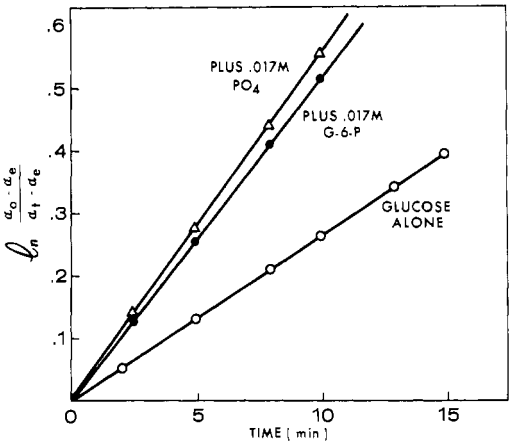

FIGURE 5: Mutarotation of glucose was measured at 25° both in EDTA buffer (5 mM, pH 7.8) and in the same buffer to which either G-6-P or P_i had been added to give final concentrations of 16.6 mM. As shown here, the mutarotation of glucose was accelerated by G-6-P to the same extent as by an equal concentration of P_i.

coefficient is some 60 times greater than that of HPO_4^{2-} (Table II). In addition, at this pH, the concentration of the potential proton donors, H_3PO_4 and $H_2PO_4^-$, is less than 0.1% of the total, thus accounting for the second-order dependence upon phosphate ion concentration.

The intramolecularly catalyzed mutarotation of G-6-P by the phosphate group at C-6 is probably not related to an increase in the *P* factor in the Arrhenius expression as was noted above. This factor would be expected to include any favorable orientation of the phosphate group at C-1. The possibility that the phosphate-catalyzed mutarotation of glucose may be enhanced by some type of complex formation between the hydroxyls on C-6 and C-1, similar to the complexes which may be formed in borate buffer, was however tested. The mutarotation of a number of sugars including D-xylose, D-ribose, and D-fucose, which have no free hydroxyl group at carbon 6, and in which preferential concentration of the phosphate group cannot therefore take place, was found to be accelerated to approximately the same degree by phosphate as were glucose and galactose (Table III).

The mutarotation of glucose was also studied in arsenate and pyrophosphate buffers of different pH values. These compounds also accelerated mutarotation to the same approximate degree as phosphate although with marked differences in the relative catalytic coefficients (Table II). The relationship between catalytic coefficient and the nature of the ions confirmed the general acid–base catalyzed nature of the reaction.

Finally it was shown (Figure 5) that G-6-P itself, when added to a mutarotating solution of α-D-glucose, accelerates the reaction to the same extent as the same molarity of P_i of the same pH value.

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