Solvolysis of Phosphoric Acid Esters. Hydrolysis of Glucose 6-Phosphate. Kinetic and Tracer Studies

Ch. Degani and M. Halmann

Contribution from the Isotope Department, The Weizmann Institute of Science, Rehovoth, Israel. *Received April 4, 1966*

Abstract: The rate of hydrolysis of D-glucose 6-dihydrogen phosphate was measured in aqueous solution at 72–100° from pH 1 to 9.6, in strongly acid solutions up to 8 *M* perchloric acid, and in strongly basic solutions up to 5 *M* sodium hydroxide. The first and second acid dissociation constants of glucose 6-dihydrogen phosphate were determined as a function of temperature. At 20°, $pK_1 = 1.50$ and $pK_2 = 6.22$. For 100° the extrapolated values are $pK_1 = 1.76$ and $pK_2 = 6.66$. From the measured rate constants and p*K* values, specific rate constants for hydrolysis of the conjugate acid, the neutral molecule, and the monoanion were determined. In strongly acid solutions at constant ionic strength, the rate of hydrolysis is proportional to the hydrogen ion concentration. On the basis of the Zucker–Hammett hypothesis, this indicates the participation of a water molecule in the rate-determining step. A similar conclusion was reached by applying the criterion of Bunnett to the acid-calalyzed reaction. Using ^{18}O-enriched water, hydrolyses of the neutral molecule and the monoanion were found to involve complete P–O bond fission, while the dianion was found to eliminate orthophosphate, mainly with C–O bond fission. In 1 to 5 *M* sodium hydroxide, the rate of release of orthophosphate is proportional to the hydroxide ion concentration. In strongly acid solution and up to pH 6.7, the rate of disappearance of glucose 6-phosphate measured by glucose 6-phosphate dehydrogenase is equal to the appearance of orthophosphate. This proves that hydrolysis is direct and does not involve some other side reactions. On the other hand, above pH 7, the rate of disappearance of glucose 6-phosphate is larger than the appearance of orthophosphate. In strongly acid solutions, glucose 6-phosphate undergoes rapid reversible ring closure to a cyclic sugar phosphate with P–O bond fission, observed through its oxygen exchange with the solvent, which is followed by the slower hydrolysis of glucose 6-phosphate with C–O bond fission. In 6 *M* $HClO_4$ at 100° the half-times of oxygen exchange and hydrolysis are 42 and 104 min, respectively.

Hexose phosphates are essential intermediates of carbohydrate metabolism. However, only for α-D-glucose 1-dihydrogen phosphate has a careful study of the mechanism of hydrolytic reactions in aqueous solutions been carried out.[1] For glucose 6-dihydrogen phosphate, only a few isolated and conflicting data on the rate of hydrolysis in acid solution have been reported.[2] While the phosphate group in glucose 1-phosphate is attached to the hemiacetal hydroxyl group, in glucose 6-phosphate it is bonded to a primary alcoholic group. This difference in structure can be expected to have important, and not easily predictable, influences on the rates and mechanisms of the hydrolytic reaction. The purpose of the present work is to determine the mechanism of hydrolysis of the various charge stages of glucose 6-phosphate, and to compare these with available data on other sugar phosphates.

Results

A. Acid Dissociation Constants. Values of the acid dissociation constants K_1 and K_2 for glucose 6-dihydrogen phosphate at 100° were required for evaluation of specific rate constants for the neutral and the various charged species, using the measured rate data at 100°. Since direct determination of acid dissociation constants at such a relatively high temperature is difficult, the extrapolation procedure of Harned and Embree[3] was applied. These authors found that the dissociation constants *K* of weak acids or bases in aqueous solution at atmospheric pressure have a maximum value K_θ in the temperature range 0–60°, and that the temperature θ of this maximal K_θ is given by

$$\log K - \log K_\theta = -p(t - \theta)^2 \qquad (1)$$

where *t* is the temperature at which the dissociation constant is *K* and *p* has the constant value 5×10^{-5}. This equation can be rearranged to the form

$$\log K + pt^2 = (\log K_\theta - p\theta^2) + (2p\theta)t \qquad (2)$$

From the linear plot of $\log K + pt^2$ against the temperature *t*, the slope $(2p\theta)$ and the intercept $(\log K_\theta - p\theta^2)$ can be obtained. From these, both the temperature θ and the maximal dissociation constant K_θ are derived. Inserting these two values in eq 1, the dissociation constant at any temperature can be calculated.

The first dissociation constant of glucose 6-dihydrogen phosphate was determined by pH measurements in solutions containing weighed amounts of glucose 6-disodium phosphate and added hydrochloric acid.

Concentrations of glucose 6-phosphate were in the 0.01 *M* range. Results at temperatures from 10 to 50°, calculated by the equation

$$pK_k = \text{pH} + \log [(C_{\text{acid}} - C_{H^+})/(C_{\text{salt}} + C_{H^+})] \qquad (3)$$

are presented in Table I. Each result is the average of 12 measurements.

The second dissociation constant was determined by potentiometric titration of a 0.02 *M* solution of disodium glucose 6-phosphate with 1.3 *N* hydrochloric

(1) C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, 3588 (1958).
(2) (a) R. Robison and E. J. King, *Biochem. J.*, **25**, 323 (1931); R. Robison, *ibid.*, **26**, 2191 (1932); (b) H. G. Hers, H. Beaufays, and C. de Duve, *Biochim. Biophys. Acta*, **11**, 416 (1953).
(3) H. S. Harned and N. D. Embree, *J. Am. Chem. Soc.*, **56**, 1050 (1934).

Table I. Temperature Dependence of pK_1 and pK_2 of Glucose 6-Dihydrogen Phosphate

	Temp, °C				
	10.3	20.0	30.5	40.1	50.0
pK_1	1.49 ± 0.03	1.50 ± 0.03	1.54 ± 0.02	1.61 ± 0.02	1.67 ± 0.02
pK_2	6.22 ± 0.02	6.22 ± 0.02	6.24 ± 0.02	6.28 ± 0.02	6.31 ± 0.02

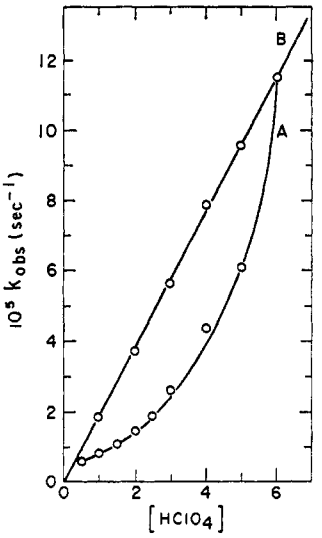


Figure 1. Rate of hydrolysis of glucose 6-dihydrogen phosphate at 100° as a function of perchloric acid concentration, without control of ionic strength (A), and at ionic strength μ = 6 (B).

acid. Henderson's equations for weak acids were used,[4] $pK_2 = pH + \log [q'/(q - q')]$, where q is the volume of added hydrochloric acid at the second equivalence point (titration of 1 equiv of sodium ion), q' the volume of added acid at any point before the equivalence point. Therefore, q' and $(q - q')$ are proportional to the concentrations of the monoanion and the dianion, respectively. Each result, as shown in the last row of Table I, is the average of 12 pH determinations.

Plots of log $K + pt^2$ against t for K_1 and K_2 were found to be linear. From the straight lines fitted to the points by a least-squares procedure, the slopes and intercepts were derived. From these, the parameters θ and K_θ of eq 1 were obtained and from these, the values of pK_1 and pK_2 at 100°.

	θ, deg	pK_θ	pK_{100}
First dissociation	18.6 ± 4.4	1.43 ± 0.02	1.76
Second dissociation	6.6 ± 1.8	6.22 ± 0.01	6.66

B. Rates of Hydrolysis. The hydrolysis of dihydrogen glucose 6-phosphate is acid catalyzed. Results for strongly acid solutions at 100° are presented in Table II. As shown in Figure 1 (plot A), the observed first-order rate constants curve up with increasing acid concentration. However, in strongly acid solutions at constant ionic strength, the rate of hydrolysis increases linearly with the hydrogen ion concentration (see Table III and Figure 1, plot B).

In moderately acid solutions, the rate of hydrolysis increases from pH 1 to 3.5, has a plateau between pH 3.5 and 5, and further increases at higher pH (see Table IV and Figure 2). In strongly alkaline solution, the rate of hydrolysis increases linearly with the hydroxide ion concentration (see Table V and Figure 3). Owing to the considerable alkaline catalysis, the half-time of the reaction in 5 M sodium hydroxide at 52.4° is only 36.5 min.

(4) A. Albert and E. P. Serjeant, "Ionization Constants of Acid and Bases," John Wiley and Sons, Inc., New York, N. Y., 1962.

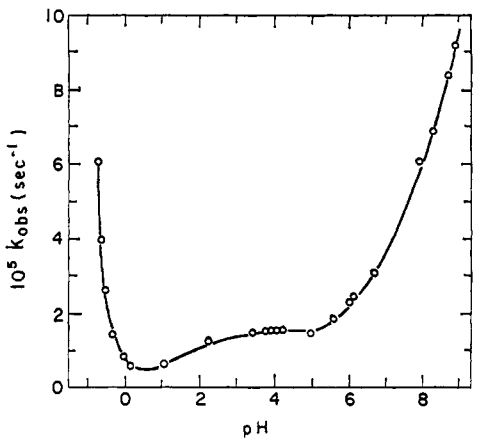


Figure 2. Rate of hydrolysis of glucose 6-phosphate at 100° as a function of pH, measured by appearance of orthophosphate.

Table II. First-Order Rate Constants for Hydrolysis of Glucose 6-Dihydrogen Phosphate in Strongly Acid Solutions at 100°

Acid, M	$10^{-5}k_{obsd}$, sec^{-1}	$10^5(k_{obsd} - k_N)$, sec^{-1}	Log a_{H_2O}
1[a]	0.806	0.426	−0.018
1.5[a]	1.09	0.71	−0.03
2[a]	1.41	1.03	−0.043
2.5[a]	1.86	1.50	−0.06
2.5[a]	1.89		
2.51[b]	2.78		
2.51[b]	2.76[c]		
3[a]	2.58	2.20	−0.08
4[a]	4.34	3.96	−0.135
5[a]	6.07	5.69	−0.215
6[a]	11.10	10.72	−0.330
7[a]	15.45	15.07	−0.496
8[a]	30.3	29.92	−0.714

[a] $HClO_4$. [b] HCl. [c] Reaction followed enzymatically with glucose 6-phosphate dehydrogenase. In all other runs, the release of orthophosphate was followed.

Table III. Rates of Hydrolysis of Glucose 6-Dihydrogen Phosphate at 100° in Strongly Acid Solutions at Constant Ionic Strength (μ = 6)

$HClO_4$, M	$NaClO_4$, M	10^5k, sec^{-1}
1.00	5	1.87
2.00	4	3.69
3.00	3	5.65
4.00	2	7.82
5.00	1	9.56
6.00	..	11.5

Table IV. Rates of Hydrolysis of Glucose 6-Phosphate at 100° as a Function of pH

pH	Buffer solution (*M*)	Method of analysis[a]	$[H_2A]/C$	$[HA^-]/C$	$[A^{2-}]/C$	$10^5 k_{obsd}$, sec^{-1}
1.05	$HClO_4$ (0.10)	P	0.836	0.164		0.561
2.25	HCl (0.134)–KCl (0.1)	P	0.245	0.755		1.28
3.45	KHP[b] (0.10)–HCl (0.0258)	P	0.020	0.980		1.495
3.80	KHP (0.25)–HCl (0.0315)	P	0.010	0.989	0.002	1.51
4.00	KHP (0.25)–HCl (0.015)	P	0.006	0.991	0.003	1.5
4.10	KHP (0.10)	P	0.005	0.992	0.004	1.52
4.23	KHP	E				1.57
4.23	KHP (0.05)	P	0.003	0.993	0.023	1.52
5.02	KHP (0.25)–NaOH (0.055)	P				1.47
5.65	KHP (0.05)–NaOH (0.0236)	P				1.85
6.05	KHP (0.05)–NaOH (0.0388)	P				2.35
6.15	Na barbitone (0.0552)–HCl (0.0478)	P				2.47
6.7	KHP (0.05)–NaOH (0.047)	P				3.02
6.7	KHP (0.05)–NaOH (0.047)	E				3.13
7.9	Na barbitone (0.087)–HCl (0.0177)	P				6.06
8.3	Na barbitone (0.0936)–HCl (0.0064)	P				6.9
8.7	Na barbitone (0.0974)–HCl (0.0026)	P				8.42
8.7	Na barbitone (0.0974)–HCl (0.0026)	E				16.8
8.85	Na barbitone (0.0985)–HCl (0.0015)	P				9.21
9.35	$NaHCO_3$ (0.05)–NaOH (0.01)	P				27.1
9.5	$NaHCO_3$ (0.05)–NaOH (0.015)	P				36.4
9.5	$NaHCO_3$ (0.05)–NaOH (0.015)	E				97.4
9.65	$NaHCO_3$ (0.05)–NaOH (0.0214)	P				49.2

[a] Methods of analysis: P = colorimetric orthophosphate determination; E = enzymatic, with glucose 6-phosphate dehydrogenase. [b] KHP = potassium hydrogen phthalate.

Table V. Rates of Hydrolysis of Disodium Glucose 6-Phosphate in Strongly Alkaline Solutions at 54.2°, Determined by Glucose 6-Phosphate Dehydrogenase (Enzymatic) and Appearance of Orthophosphate (PO_4^{3-})

NaOH, *M*	$10^4 k_{obsd}$, sec^{-1} (enzym)	$10^4 k_{obsd}$, sec^{-1} (PO_4^{3-})
1		1.46
2		1.93
2.5	5.02	2.10
3		2.31
4		2.67
5		3.17

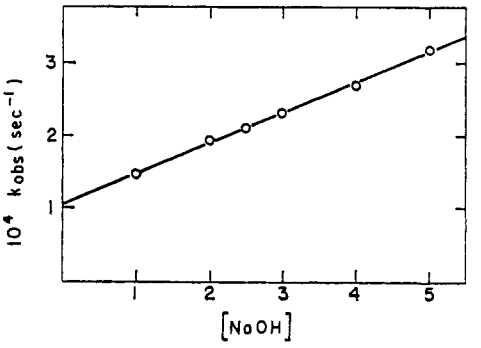


Figure 3. Plot of hydrolysis of glucose 6-disodium phosphate at 54.2° as a function of sodium hydroxide concentration.

Rate measurements in strongly alkaline solutions (above 1 *N* NaOH) were carried out under a nitrogen atmosphere in order to remove oxygen, in the presence of which alkali leads to rapid degradation of sugars with formation of a complex mixture of products.[5] In weakly basic medium no oxygen effect was found. At pH 8.85, the same rate of hydrolysis was found in solutions in evacuated sealed tubes, and in sealed tubes containing air at atmospheric pressure (9.17×10^{-5} and 9.21×10^{-5} sec^{-1}, respectively, at 100°).

The rate $k = 0.805 \times 10^{-5}$ sec^{-1} obtained for hydrolysis in 1 *M* perchloric acid is similar to the rate in 1 *M* hydrochloric acid as calculated from the percentage of hydrolysis after 3 hr ($k = 0.94 \times 10^{-5}$).[2b]

(5) L. F. Leloir and C. E. Cardini in "Comprehensive Biochemistry," Vol. 5, M. Florkin and G. H. Stotz, Ed., Elsevier Publishing Co., Amsterdam, 1963.

The rate of hydrolysis was followed by two methods: (a) colorimetric determination of orthophosphate produced in the course of hydrolysis, and (b) enzymatic essay of the residual glucose 6-phosphate. In strongly acid solutions and up to pH 7, it was found (see Table IV) that the rate of formation of orthophosphate equals the rate of disappearance of glucose 6-phosphate, as determined by glucose 6-phosphate dehydrogenase. In more basic solutions, the rate of disappearance of glucose 6-phosphate is faster than the rate of appearance of orthophosphate. This may indicate that while in the strong and weak acid media the main reaction is hydrolysis, in the more basic media the glucose 6-phosphate undergoes some other side reaction to form an intermediate, which then more slowly releases the orthophosphate. In order to compare the results ob-

Table VI. Points of Bond Fission in Hydrolysis of Glucose 6-Phosphate, at 100°, Determined in ^{18}O-Enriched Water (Initially Containing N_S At. % Excess ^{18}O) Forming Orthophosphate (Containing N_P At. % Excess ^{18}O)

pH	Time, hr,	N_S	N_P	$4N_P/N_S$ (fraction of P–O bond fission)	No. of O atoms exchanged
2 *N* NaOH[a]		16.1	0.04	0.01	
6.25–7.1	5.25	25.7 ± 1.3	1.04 ± 0.09	0.162	
6.35–6.9	2.75	12.6 ± 0.2	0.454 ± 0.008	0.144	
6.25–6.7	3.25	15.3	0.525	0.137	
6.23–6.65	3.25	15.55 ± 0.05	0.572	0.148	
4.07–4.35	12	15.45 ± 0.10	4.01	1.04	
1.6–2	27.5	15.13 ± 0.06	3.79 ± 0.02	0.99	
$HClO_4$					
6 *M*	0.5	24.81 ± 0.2	11.65		1.88
6 *M*	1.5	24.81 ± 0.2	16.8 ± 0.05		2.71
6 *M*	2.083	24.81 ± 0.2	17.25		2.78
6 *M*	2.5	24.81 ± 0.2	17.63 ± 0.1		2.85
6 *M*	3.5	24.81 ± 0.2	18.7 ± 0.3		3.01
6 *M*	1	24.7 ± 0.2	14.1 ± 0.1		2.28
6 *M*	4	24.7 ± 0.2	17.97 ± 0.35		2.92
6 *M*	7	24.7 ± 0.2	18.55 ± 0.4[b]		3.00[b]
6 *M*	16	24.7 ± 0.2	18.83 ± 0.3[b]		3.05[b]

[a] At 52.4°. [b] These values have been corrected for the isotopic exchange of orthophosphate with the water, according to C. A. Bunton, D. R. Llewellyn, C. A. Vernon, and V. A. Welch, *J. Chem. Soc.*, 1636 (1961).

tained by both methods in strongly acid solutions, we had to carry out the hydrolysis in hydrochloric acid instead of perchloric acid because perchlorate is known to inhibit the enzymatic activity of glucose 6-phosphate dehydrogenase.[2b]

C. Point of Bond Fission. Hydrolysis of glucose 6-dihydrogen phosphate, or its various ionic forms, may occur either with P–O or with C–O bond fission. Using ^{18}O-labeled water, the isotopic enrichment in the resulting orthophosphate from hydrolysis in weakly acid solutions was found to be one-fourth that of the solvent, as shown in Table VI. Therefore, in weakly acid media, hydrolysis involves essentially only P–O bond fission. In neutral and basic solutions, on the other hand, almost complete C–O bond fission was observed.

In these experiments, unbuffered solutions were used. The pH data listed in Table VI represent the initial and final measured values.

D. Oxygen Exchange in Strongly Acid Solutions. In strongly acid media, hydrolysis in ^{18}O-enriched water was accompanied by rapid isotopic exchange of glucose 6-phosphate with the solvent. From the linear plot of log (1 − fraction of exchange) *vs.* time, the half-life of the exchange was found to be 42 min (at 100° in 6 *M* perchloric acid, see Table VI). The half-life for hydrolysis under these conditions was 104 min. The incorporation of ^{18}O into the glucose 6-phosphate did not exceed three out of the four oxygen atoms of the phosphate group. After hydrolysis, the resulting orthophosphate had an ^{18}O excess equal to three-fourths that of the solvent. Thus it is necessary to conclude that hydrolysis occurs with C–O bond fission, and that it is preceded by rapid reversible exchange with P–O bond fission.

E. Temperature Effects. Rates of hydrolysis of various charge states of glucose 6-phosphate at several temperatures are presented in Table VII. From Arrhenius plots of log k *vs.* $1/T$, the activation energies E_a were obtained, and from these by the equation[6]

$$k = (ekT/h)(e^{\Delta S^*/R})e^{-Ea/R}$$

values for the entropies of activation ΔS^*. Results are included in Table VII.

Table VII. Temperature Dependence of the Rates of Hydrolysis of Glucose 6-Phosphate in Different Media

Solvent	Temp, °C	$10^6 k_{obsd}$, sec^{-1}	E_a, kcal $mole^{-1}$	ΔS^*, eu at 100°
$HClO_4$ (6 *N*)	72.1	4.33		
	87	25.3		+0.18
	100	115	29.3 ± 0.2	
pH 4	72.1	0.427		
	87	2.58		+2.33
	100	15.2	32.1 ± 0.1	
pH 6.7	72.1	0.745		
	87	5.69		+8.24
	100	30.2	33.5 ± 0.1	

Discussion

The pH dependence of the rate of hydrolysis is presented in Figure 2.

Analysis of the kinetic data must involve consideration of the specific reactivity of the various charge states of glucose 6-phosphate, the conjugate acid H_3A^+, the neutral acid H_2A, the monoanion HA^-, and the dianion A^{2-}, all of which are in rapid acid–base equilibrium.

$$H_3A^+ \overset{H_3O^+}{\rightleftharpoons} H_2A \overset{K_1}{\rightleftharpoons} HA^- + H^+ \overset{K_2}{\rightleftharpoons} A^{2-} + H^+$$
$$\downarrow k_A \qquad \downarrow k_N \qquad \downarrow k_1 \qquad \downarrow k_2$$

From the acid dissociation constants K_1 and K_2, it is possible to calculate the mole fractions of the neutral molecule and of the mono- and dianions in moderately acid solutions (pH 1 to 5). The results are given in Table IV. Since in a solution of any given pH, only two charge states of the substrate can be predominant, one can (assuming all charge states undergo pseudo-unimolecular hydrolysis) describe the observed first-

(6) S. Glasstone, K. J. Laidler, and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Co. Inc., New York, N. Y., 1941, p 199.

order rate constant as a function of the concentration of one of the charge states.

A. Acid Dissociation Constants. The first dissociation constant found in the present work for 20°, pK_1 = 1.50, differs considerably from that previously reported[7] for glucose 6-dihydrogen phosphate at 21°, pK_1 = 0.94, which has been quoted even in recent reviews.[5]

The previous work was, however, carried out in rather concentrated solutions (0.1 *M*) without applying activity coefficient corrections. In the present measurements, made in more dilute solutions (0.01 *M*), such corrections are negligible.[4]

The present value for glucose 6-phosphate is larger than that reported for glucose 1-phosphate (pK_1 = 1.23 at 30°),[1] whereas the corresponding value reported in the literature for glucose 6-phosphate is lower than that found for glucose 1-phosphate.

The pK_1 of glucose 1-phosphate may be expected to be lower than that of glucose 6-phosphate because of inductive effects. These may be divided into two parts.

(a) The Inductive Effect of the Oxygen in the Sugar Ring.[8] The phosphate hydroxyl of glucose 1-phosphate is located closer to the oxygen of the sugar ring (a distance of three atoms) than the phosphate hydroxyl of glucose 6-phosphate (a distance of four atoms). An estimate of the increase in acidity is obtained by use of the inductive constants of Branch and Calvin.[9] The inductive effect of an oxygen atom three atoms removed over one four atoms removed will make the acidity of glucose 1-phosphate stronger by 0.12 pK_a unit.[8]

(b) The Inductive Effect of a Neighboring Hydroxyl Group. While the phosphate group of glucose 1-phosphate has a neighboring hydroxyl group in the β position, glucose 6-phosphate has a neighboring hydroxyl group only in the γ position to the phosphate group. It is obvious that the electron-withdrawing effect of a hydroxyl group in the β position is more pronounced and, accordingly, glucose 1-phosphate should be the more acidic.

An estimate of the decrease in the inductive effect of a hydroxyl group bonded to the γ position in comparison with that of a β-substituted hydroxyl group can be obtained from the observed pK values of β- and γ-hydroxybutyric[10] acids. These indicate that a shift of a hydroxylic group from the β to the γ position involves a decrease of 0.2 pK_a unit.

Considering the combined contribution of both effects (0.12 + 0.2) we may expect a difference of 0.32 pK_a unit between glucose 1-phosphate and glucose 6-phosphate. The value for pK_1 of glucose 6-phosphate as measured at 30° (1.54) is in good agreement with the expected value (1.23 + 0.32 = 1.55).

In order to rationalize the supposedly greater acid strength of glucose 6-phosphate over glucose 1-phosphate, as determined by Meyerhof and Lohmann,[7] Kumler and Eiler[8] invoked the possible formation of chelate rings of different sizes in the two compounds. According to these authors, glucose 1-phosphate has the acetal oxygen in the most suitable position for

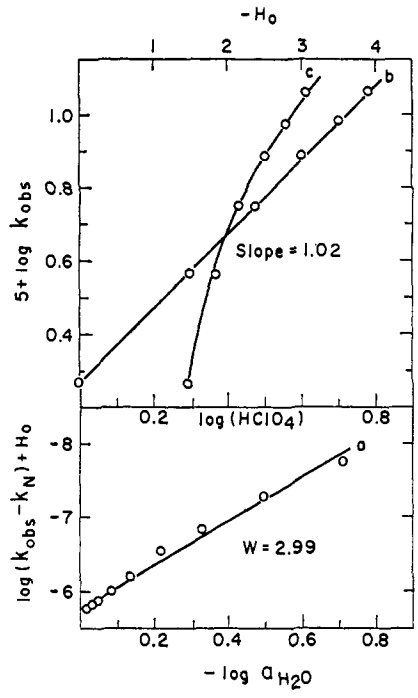


Figure 4. Plots of (a) log ($k_{obsd} - k_N$) + H_0 against log a_{H_2O}, (b) log k_{obsd} against log [$HClO_4$], and (c) log k_{obsd} against H_0 for hydrolysis of glucose 6-dihydrogen phosphate at 100°. Plots b and c are at μ = 6.

binding with a phosphoric acid hydroxyl by hydrogen bonds. Such type of bonding is considered to be acid weakening because it decreases the dissociation of the phosphoric acid hydroxyl. In glucose 1-phosphate a six-membered ring can be formed while in glucose 6-phosphate only the less-stable seven-membered ring is possible. The effect of the latter is therefore less pronounced. It seems to us, however, that this argument can explain the difference in pK_2 values and not in pK_1 values, and indeed we find that glucose 6-phosphate has a lower pK_2 than glucose 1-phosphate[11] (6.24 and 6.51, respectively, at 30°).

B. The Acid-Catalyzed Reaction. The rate of hydrolysis of dihydrogen glucose 6-phosphoric acid in simple mixtures of perchloric acid and water does not increase linearly with the acid concentration (see Figure 1). Part of the observed enhancement in rate must be due to the increased ionic strength of solutions containing larger proportions of perchloric acid. Therefore, it is preferable to determine the rate of hydrolysis as a function of acid concentration at constant ionic strength.[12] From the slope of the linear plot of k_{obsd} against the perchloric acid concentration, we get for the rate of the second-order acid-catalyzed reaction (at 100° and ionic strength μ = 6), k_A = (1.94 ± 0.02)10^{-5} l. $mole^{-1}$ sec^{-1}. This is an order of magnitude larger than the rate reported for methyl dihydrogen phosphate,[12] 3.08 × 10^{-6} l. $mole^{-1}$ sec^{-1}.

As shown in Figure 4, the observed first-order rate constant is proportional, on a log–log scale, to the con-

(7) O. Meyerhof and K. Lohmann, *Biochem. Z.*, **185**, 113 (1927).
(8) W. D. Kumler and J. J. Eiler, *J. Am. Chem. Soc.*, **65**, 2355 (1943).
(9) G. G. K. Branch and M. Calvin, "The Theory of Organic Chemistry," Prentice-Hall, Inc., New York, N. Y., 1961.
(10) "International Critical Tables," Vol. VI, McGraw-Hill Book Co., Inc., New York, N. Y., 1929, p 259.
(11) (a) W. E. Trevelyan, P. F. E. Mann, and J. S. Harrison, *Arch. Biochem. Biophys.*, **39**, 419 (1952); (b) J. H. Ashby, H. B. Clarke, E. M. Crook, and S. P. Datta, *Biochem. J.*, **59**, 203 (1955).
(12) C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, 3574 (1958); C. A. Bunton, D. R. Llewellyn, C. A. Vernon, and V. A. Welch, *ibid.*, 1636 (1961); C. A. Bunton, M. M. Mhala, K. G. Oldham, and C. A. Vernon, *ibid.*, 3293 (1960).

centration of perchloric acid, with a slope of 1.02 ± 0.01. The rate is not linearly related to the Hammett acidity function. According to the Zucker–Hammett hypothesis,[13] this indicates the participation of a water molecule in the rate-determining step.

Another approach to the study of the mechanism of hydrolysis in strongly acid solutions has been proposed by Bunnett.[14] In order to apply this approach, it is necessary to deduct from the observed first-order rate constant, k_{obsd}, the contribution of the rate k_N of the nonacid-catalyzed hydrolysis of neutral glucose 6-dihydrogen phosphoric acid.

From the linear plot of log $(k_{obsd} - k_N) + H_0$ against $-\log a_{H_2O}$, where a_{H_2O} is the activity of water in the solvent, a slope $w = 2.99 \pm 0.13$ is obtained by a least-squares fit, using Yates and Wai's values for H_0.[15] (Using previous values for H_0,[12] we get $w = 3.33 \pm 0.15$). This value of w indicates that the acid-catalyzed hydrolysis of glucose 6-dihydrogen phosphate belongs to the group of reactions with a rate-determining step involving a water molecule as the nucleophilic reagent.[14] Thus, both the Zucker–Hammett and the Bunnett approach lead to the same mechanistic conclusion.

In contrast, by the same criteria, the acid-catalyzed hydrolysis of glucose 1-dihydrogen phosphate does not involve a water molecule in the rate-determining step, and may thus be considered a unimolecular reaction.[1]

Further information about the mechanism of the reaction is gained by consideration of the actual magnitudes of the activation parameters. In strongly acid solutions we measured a value of ΔS equal to 0.18 eu. By comparison of this value to those obtained for acid-catalyzed hydrolysis of glycosides[16a] (mean value 13.7 eu), for which a "unimolecular" mechanism was proposed (because of observed proportionality between rate and Hammett's acidity function, as well as solvent deuterium isotope effect), we can see that the entropy of activation of glucose 6-phosphate hydrolysis is considerably lower than those of glycosides. Such a difference can be associated with a different reaction mechanism, a more negative entropy suggesting a more highly ordered transition state. Thus while glycosides hydrolyze *via* a unimolecular (A1) mechanism, the hydrolysis of glucose 6-phosphate seems to be bimolecular.

However, it should be mentioned that in the case of sugars the entropy criterion is subject to some uncertainty because of the possibility of large structural or solvation effects.[16b]

C. Oxygen Exchange in the Phosphate Group in Glucose 6-Phosphate in Strongly Acid Solutions. Migration of the phosphate group in glucose 6-phosphate in strongly acid media is similar to that observed previously in 1- and 2-glycerophosphate, in sugar alcohol phosphates, and in sugar phosphates.[17]

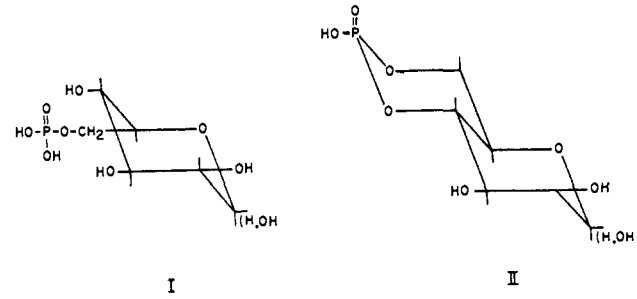
Glucose 6-phosphate exists in the pyranose form (stable C-1 conformation), and cyclization may occur between the phosphate group and the equatorial hydroxyl function on C-4 leading to a six-membered cyclic phosphate.

The isotope exchange in I is considered to proceed through formation of such a cyclic intermediate II which breaks up rapidly with P–O bond fission only. The rate of isotopic exchange in glucose 6-phosphate is less than that found for 1,2 propanediol 1-dihydrogen phosphate.[17b] This is in accord with the known fact[17f] that five-membered cyclic phosphates are more labile to acid hydrolysis than the six-membered ones.

Cyclization in glucose 6-phosphate may also involve the hydroxyl group in the 3 position forming a seven-membered cyclic phosphate. Formation of such a ring is not favored sterically.[17g] Moreover its stability to acid is much higher than that of the six-membered ring. The latter is therefore more likely to participate as an intermediate in the phosphate group migration.

An alternative mechanism for the oxygen exchange in the phosphate group would be rapid reversible addition of water to the phosphoryl bond, to form a pentacovalently bonded phosphorus intermediate. However, since in alkyl dihydrogen phosphates under similar conditions no appreciable oxygen exchange could be detected, it seems necessary to conclude that the rapid exchange in glycero phosphates and glucose 6-phosphate is due to interaction of the phosphoryl group with one of the hydroxyl groups on the carbon chain.

It should also be mentioned that in α-D-glucose 1-phosphate, which exists in the stable C-1 conformation, cyclization may occur between the axial phosphate group and the equatorial hydroxyl group in position 2 (*cis*), forming a five-membered ring which is very labile to acid.[17f] However, isotopic experiments carried out by Vernon, *et al.*,[1] exclude the possible occurrence of such a migration in glucose 1-phosphate.



I II

Parallel with this exchange, but at a slower rate, glucose 6-phosphate undergoes hydrolysis, with C–O bond fission only, presumably by a bimolecular mechanism, with a water molecule participating in the rate-determining step. Attack of the water molecule must therefore be on the carbon atom of the ester bond of the conjugate acid (the protonated form) of the sugar phos-

(13) F. A. Long and M. A. Paul, *Chem. Rev.*, 57, 935 (1957). However, see also criticism of this hypothesis by E. Whalley, *Advan. Phys. Org. Chem.*, 2, 93 (1964).

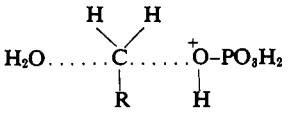
(14) J. F. Bunnett, *J. Am. Chem. Soc.*, 83, 4956, 4968, 4973, 4978 (1961).

(15) K. Yates and H. Wai, *ibid.*, 86, 5408 (1964).

(16) (a) W. G. Overend, C. W. Rees, and J. S. Sequeira, *J. Chem. Soc.*, 3429 (1962); (b) L. L. Schaleger and F. A. Long, *Advan. Phys. Org. Chem.*, 1, 1 (1963).

(17) (a) P. W. C. Barnard, C. A. Bunton, D. R. Llewellyn, K. G. Oldham, B. L. Silver, and C. A. Vernon, *Chem. Ind.* (London), 760 (1955); (b) L. Kugel and M. Halmann, *J. Am. Chem. Soc.*, 88, 3566 (1966); (c) F. L. Pizer and C. E. Ballou, *ibid.*, 81, 915 (1959); (d) J. Baddiley, J. G. Buchanan, and B. Carss, *J. Chem. Soc.*, 1869 (1957); (e) J. Baddiley, J. G. Buchanan, and L. Szabo, *ibid.*, 3826 (1954), giving further references; (f) H. G. Khorana, G. M. Tener, R. S. Wright, and J. G. Moffat, *J. Am. Chem. Soc.*, 79, 430 (1957); (g) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961, p 62.

phate. The transition state may be represented by the following representation.



D. The Neutral Species and the Monoanion. In the pH range 1–4, only the neutral molecule (mole fraction $[H_2A]$) and the monoanion (mole fraction $[HA^-]$) must be considered.

$$\begin{aligned} k_{obsd} &= k_1[HA^-] + k_N[H_2A] \\ &= k_1[HA^-] + k_N(1 - [HA^-]) \\ &= (k_1 - k_N)[HA^-] + k_N \end{aligned}$$

Therefore k_{obsd} should be linearly related to $[HA^-]$, with a slope $(k_1 - k_N)$, and an intercept k_N. From a least-square fit, we get for hydrolysis of glucose 6-phosphate at 100°, $k_N = (0.38 \pm 0.01) \times 10^{-5}$ sec^{-1} and $k_1 = (1.52 \pm 0.01) \times 10^{-5}$ sec^{-1}. For k_N, the rate constant for hydrolysis of the neutral molecule, another determination can be made from the plot of k_{obsd} against the hydrogen ion concentration $[H^+]$ in *strongly* acid solutions (Figure 1). Extrapolation to $[H^+] = 0$ yields the value $k_N = 0.4 \times 10^{-5}$ sec^{-1} in agreement with the above extrapolation from *weakly* acid solutions.

The monoanion of glucose 6-phosphate is four times more reactive than the neutral molecule. The predominant reactivity of the monoanion is, therefore, less marked than for hydrolysis of methyl dihydrogen phosphate, in which the monoanion is about 17 times more reactive than the neutral molecules. On the other hand, in glucose 1-phosphate, the neutral species reacts much more rapidly than the monoanion, by a factor of 2×10^4.

The oxygen isotope experiments (Table VI) show that at pH 1.6–2.0, at which glucose 6-phosphate exists partly as the neutral molecule (as well as the monoanion), hydrolysis occurs only with P–O bond fission. The neutral molecule of glucose 6-phosphate reacts thus differently from the neutral species in methyl dihydrogen phosphate and glucose 1-phosphate, which undergo mainly C–O bond fission. Only for the hydrolysis of the monoanion, which is predominant at pH 4, the same mechanism of P–O bond fission was found for all three alkyl dihydrogen phosphates.

E. The Dianion and Alkaline Catalysis. In the pH range 5–6.7, the mono- and dianion are the predominant species. Considering the observed rate constant to be the sum of those due to the first-order hydrolysis of the monoanion and of the dianion, we get

$$\begin{aligned} k_{obsd} &= k_1[HA^-] + k_2[A^{2-}] \\ &= k_1(1 - [A^{2-}]) + k_2[A^{2-}] \\ &= k_1 + [A^{2-}](k_2 - k_1) \end{aligned}$$

From the linear plot of k_{obsd} against the mole fraction of the dianion, $[A^{2-}]$, values for the intercept and slope were obtained. At 100° the results are $k_1 = 1.52 \times 10^{-5}$ sec^{-1} and $k_2 = 5.15 \times 10^{-5}$ sec^{-1}. This value for k_1 agrees very well with that obtained above from experiments in the pH range 1–4.

The main reaction of glucose 6-phosphate below pH 7 is hydrolysis, as can be seen by following the disappearance of glucose 6-phosphate. The contribution of the reaction of the monoanion decreases as the pH rises, but the contribution of the unimolecular hydrolysis of the dianion never reaches its maximum, as indicated by the absence of a plateau between pH 8 and 10. The steady increase of reaction rate, contrary to the expected constant value in the range pH 8–10, may probably be due to some other reactions which glucose 6-phosphate may undergo, besides the unimolecular hydrolysis of the dianion. This is also supported by the enzymatic experiments in which we get a higher rate of disappearance of glucose 6-phosphate than that of orthophosphate release. The predominant C–O bond breakage (Table VI), as well as the positive value of the entropy of activation, +8.2 eu (Table VII), suggest a unimolecular mechanism for the hydrolysis of the dianion.

The mechanism of the reaction in alkaline media will be subject to further investigation.

In contrast to the alkali stability of aldose 1-phosphates, such as glucose 1-phosphate, sugar phosphates having a free aldehyde group are subject to alkaline-catalyzed hydrolysis. A β elimination mechanism has been proposed[18] to account for such a reaction.

Experimental Section

Materials. Disodium D-glucose 6-phosphate (Sigma) and dipotassium D-glucose 6-phosphate (Fluka) were used without further purification. The disodium salt had $[\alpha]^{24}D$ 23.26°. The dipotassium salt had $[\alpha]^{24}D$ 23.3° (lit.[19] 21.2°). *Anal.* Calcd for $Na_2C_6H_{11}PO_9$: C, 21.2; H, 4.4; P, 9.12. Found: C, 21.4; H, 4.8; P, 9.08.

Buffer Solutions. The pH of the buffer solutions which were used in the kinetic experiments was measured at 95°, using a Radiometer TTT1a pH meter fitted with a temperature compensator. For several of these buffers, data on their pH at 20 and 150° were reported by Stene.[20] Simple linear interpolation of these data yielded for 100° values which agreed well with our measured pH at 95°.

Acid Dissociation Constants of glucose 6-dihydrogen phosphate were determined by potentiometric titration, using the Radiometer pH meter, fitted with a scale expander, which permitted an accuracy of ±0.01 pH unit. The titration cell was a double-walled beaker, through the jacket of which thermostated water (±0.1°) was circulated.

Rate Measurements. (1) Acid Solutions. Solutions of glucose 6-phosphate (about 2 ml) in sealed glass tubes were kept in a thermostat. At various intervals, tubes were taken out and cooled in ice–water. The orthophosphate produced was analyzed by the molybdate method,[21] using a Unicam SP600 spectrophotometer to determine the absorptivity at 660 mμ.

Disappearance of glucose 6-phosphate was followed by its enzymatic determination using glucose 6-phosphate dehydrogenase (Sigma Chemical Co.), in the presence of triphosphopyridine nucleotide (TPN, Sigma Chemical Co). The appearance of the reduced TPN was measured spectrophotometrically at 340 mμ.[22]

(2) Strongly Alkaline Solutions. Solutions of glucose 6-phosphate were placed in polyethylene test tubes and nitrogen was bubbled through for a few minutes to remove oxygen. The tubes were closed with polyethylene stoppers and thermostated.

(3) Assay of Glucose 6-Phosphate was made by periodate oxidation.[23] A test tube containing 1 ml of the sugar phosphate solution, 1 ml of concentrated sulfuric acid, and 1 ml of periodic acid was kept for 1 hr at 100°. After cooling to room temperature, 1

(18) D. M. Brown, F. Hayes, and A. R. Todd, *Chem. Ber.*, 90, 936 (1957).
(19) H. A. Lardy and H. O. L. Fischer, *J. Biol. Chem.*, 164, 513 (1946).
(20) S. Stene, *Rec. Trav. Chim.*, 49, 1133 (1930).
(21) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, 66, 375 (1925).
(22) "Methods in Enzymology," Vol. III, S. P. Colowick and N. O. Kaplan, Ed., Academic Press Inc., New York, N. Y., 1957, p 153.
(23) C. F. Burmaster, *J. Biol. Chem.*, 164, 233 (1946); H. Paulus and E. P. Kennedy, *ibid.*, 235, 1303 (1960).

ml of 4% sodium sulfite was added to the solution to reduce the excess periodic acid and the iodic acid formed by its reduction. The solution was then neutralized with 10 *N* sodium hydroxide and analyzed for orthophosphate, as above.

Determination of the Position of Bond Breakage. Disodium glucose 6-phosphate (about 400 mg) was dissolved in ^{18}O-enriched water (about 10 ml), sealed in glass tubes, and kept at 100° to about 50% reaction. The solution was brought to room temperature; the resulting orthophosphate was precipitated as magnesium ammonium phosphate, separated by centrifugation, dissolved in hydrochloric acid, and reprecipitated with concentrated ammonia. A suspension of the precipitate in a small volume of water was shaken with an excess of Dowex 50 (ionic form H^+) ion exchanger. The resin was rinsed with ethanol, and the combined washings were brought to pH 4 (methyl orange) with 3 *N* potassium hydroxide, thus precipitating potassium dihydrogen phosphate (adding acetone to complete the precipitation). After drying at 100° under reduced pressure, the ^{18}O-content of the phosphate, as well as of the water at the completion of reaction, were determined by the method of Boyer, *et al.*,[24] with several duplicate runs being made for each sample.

Acknowledgment. This investigation was supported by Research Grant GM-05842 from the U. S. Public Health Service.

(24) P. D. Boyer, D. J. Graves, C. H. Suelter, and M. R. Dempsy, *Anal. Chem.*, 33, 1906 (1961).

The Hydrolysis of Glucose 6-Phosphate[1,2]

C. A. Bunton and Hernan Chaimovich[3]

Contribution from the Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California. *Received April 4, 1966*

Abstract: At low pH the kinetic form of the hydrolysis of glucose 6-phosphate is very similar to those found for other simple monoalkyl phosphates. There is an acid-catalyzed hydrolysis, with an A2 mechanism, and halogen acids are very effective catalysts because of nucleophilic attack of the halide ion upon carbon. The reaction rate is constant in the region pH 3–5 where the monoanion is the bulk component, but at a high pH it increases as the dianion becomes the bulk component, and there is a second plateau at pH 7–8. This dianion reaction probably involves the β anomer and occurs with participation of the 1-OH group acting as an intramolecular general acid. At pH >9 two new reactions appear, one giving a 1,6-anhydro sugar, probably by nucleophilic attack of the 1-alkoxide ion upon C-6 with expulsion of phosphate trianion. The other (major) reaction involves either attack of hydroxide ion upon the dianion of glucose 6-phosphate assisted by intramolecular general acid catalysis by the 1-OH, or attack of water assisted by the 1-alkoxide group acting as a general base. This second mechanism appears to be the more probable.

The mechanisms of the hydrolysis of monoalkyl phosphates are generally well understood.[4] At pH 3–5 the predominant species is the monoanion (I), which probably decomposes by eliminating a metaphosphate ion,[5–8] although there is some question as to the intimate details of the reactions.[9,10]

The dianion II is generally unreactive,[4–7] and it has always been assumed that the monoalkyl phosphates are unreactive toward alkali,[11] simply because electrostatic repulsion hinders attack of hydroxide ion upon the dianion.

Attack of hydroxide upon the dianion can, however, be observed with aryl phosphates,[12] and powerful electron-attracting substituents change the mechanisms of hydrolysis so much that the dianion of 2,4-dinitrophenyl dihydrogen phosphate is very reactive both toward hydroxide ion and in its spontaneous hydrolysis.[13]

The undissociated acid (III) is generally less reactive than the monoanion (I), with exceptions for compounds in which the alkyl group can separate as a carbonium ion.[5,14,15]

There may also be an acid-catalyzed hydrolysis which involves the conjugate acid (IV)[5,6,14] and becomes important only at pH <1. Therefore, generally for hydrolysis of a simple monoalkyl phosphate, one observes a rate constant which decreases with decreasing acidity, with a minimum at pH ~1, followed by an increase to a maximum at pH 3–5, where I is the bulk component. The rate then decreases at high pH as the dianion becomes the bulk component.[4]

(1) This work was supported by the National Science Foundation under Grant GP2887. This support is gratefully acknowledged.

(2) Presented in part at the Symposium on Bio-organic Mechanisms, American Chemical Society Western Regional Meeting, Los Angeles, Calif., Nov 19, 1965.

(3) Scholar of the Rockefeller Foundation on leave of absence from Facultad de Quimica y Farmacia, Universidad de Chile. This support is gratefully acknowledged.

(4) J. R. Cox and O. B. Ramsay, *Chem. Rev.*, **64**, 317 (1964).

(5) (a) W. W. Butcher and F. H. Westheimer, *J. Am. Chem. Soc.*, 77, 2420 (1955); (b) J. Kumamoto and F. H. Westheimer, *ibid.*, 77, 2515 (1955); (c) F. H. Westheimer, Special Publication No. 8, The Chemical Society, London, 1957, p 1.

(6) P. W. C. Barnard, C. A. Bunton, D. R. Llewellyn, K. G. Oldham, B. A. Silver, and C. A. Vernon, *Chem. Ind.* (London), 760 (1955); C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, 3574 (1958).

(7) C. A. Vernon, ref 5c, p 17.

(8) P. A. T. Swoboda, ref 5c p 41; J. D. Chanley and E. Feageson, *J. Am. Chem. Soc.*, **85**, 1181 (1963).

(9) W. P. Jencks and M. Gilchrist, *ibid.*, **86**, 1410 (1964).

(10) C. A. Bunton and H. Chaimovich, *Inorg. Chem.*, **4**, 1763 (1965).

(11) G. Schmidt and M. Laskowski in "The Enzymes," Vol. 5, 2nd ed, P. D. Boyer, H. Lardy, and K. Myrbäck, Ed., Academic Press Inc., New York, N. Y., 1961, Chapter 1.

(12) A. J. Kirby and W. P. Jencks, *J. Am. Chem. Soc.*, **87**, 3209 (1965).

(13) Unpublished results by E. Fendler; C. A. Bunton in Symposium on Bioorganic Mechanisms, ref 2.

(14) C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, 3588 (1958).

(15) A. Lapidot, D. Samuel, and B. Silver, *Chem. Ind.* (London), 468 (1963).