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HYDROLYSIS OF α-D-GLUCOPYRANOSE 1,2-CYCLIC PHOSPHATE: THE EFFECT OF pH AND TEMPERATURE ON THE PRODUCT DISTRIBUTION, AND THE POSITION OF OPENING OF THE PHOSPHATE DIESTER RING IN FORMATION OF D-GLUCOSE 2-PHOSPHATE

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ABSTRACT

The chemical hydrolysis of α-D-glucopyranose 1,2-cyclic phosphate to α-D-glucopyranosyl phosphate and D-glucose 2-phosphate has been studied over the pH range 5–13 at 80° and at pH 1–4 at several temperatures. At pH 1, α-D-glucopyranosyl phosphate is relatively stable at temperatures below 60°, but at higher temperatures the rate of further hydrolysis to D-glucose and inorganic phosphate is comparable to the rate of hydrolysis of the cyclic ester. At basic pH values at 80°, hydrolysis of the cyclic ester is fast relative to further hydrolysis of the monoesters produced. Results of hydrolyses of the α-D-glucopyranose 1,2-cyclic phosphate in $^{18}OH_2$ are consistent with formation of D-glucose 2-phosphate via P–O cleavage in both 0.1M sodium hydroxide and 0.1M hydrochloric acid at 100°. These results and others suggest that the ring strain proposed to account for the rapid rate of hydrolysis of five-membered cyclic phosphates does not manifest itself by accelerated cleavage of the C-1–O(P) bond of α-D-glucopyranose 1,2-cyclic phosphate.

INTRODUCTION

We describe results of experiments concerning the pH and temperature dependence of the product distribution in the hydrolysis of α-D-glucopyranose 1,2-cyclic phosphate (Glc-1,2-P)†. These experiments were originally undertaken to see whether simple conditions could be found where Glc-1-P would be the major product, as in the enzymic hydrolysis of this cyclic ester[1]. If conducted in ^{18}O-enriched water, and if it proceeded via P–O cleavage, this would allow a simple chemical synthesis

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†Abbreviations: Glc-1-P, α-D-glucopyranosyl phosphate; Glc-1,2-P, α-D-glucopyranose 1,2-cyclic phosphate; Glc-2-P, D-glucose 2-phosphate; and Pi, inorganic phosphate.

labelled Glc-1-P has been synthesized by enzymic hydrolysis of Glc-1,2-P and used by us in studies of the mechanism of potato-starch phosphorylase[2].

A second aspect of hydrolysis of Glc-1,2-P is of interest. The rapid hydrolysis of five-membered cyclic phosphate diesters has been rationalized in terms of ring-strain relief when water or hydroxide ion adds to the phosphorus atom to generate a pentacovalent intermediate[3]. We wondered if this ring strain might also be manifested as an accelerated ring-opening via C–O bond cleavage at C-1; such a C–O cleavage would be possible in this instance, because a carbonium ion at C-1 would be stabilized by the oxygen atom of the pyranose ring. Thus, the hydrolysis of Glc-1-P in acid has been shown to proceed via C–O cleavage[4].

The chemistry of Glc-1,2-P has recently taken on biochemical significance with the finding that it is a potent inhibitor of potato-starch phosphorylase[5], and is formed[6] by metal ion-catalyzed hydrolysis of uridine 5′-(α-D-glucopyranosyl diphosphate) at neutral pH and 37°.

Paladini and Leloir[7] had earlier shown that Glc-1,2-P could be obtained by alkaline cleavage of uridine 5′-(α-D glucopyranosyl diphosphate). They also found that hydrolysis of Glc-1,2-P in 0.05M sulfuric acid at 100° yielded D-glucose and Glc-2-P in the ratio 1:3, whereas hydrolysis in 0.1M sodium hydroxide yielded Glc-1-P and Glc-2-P in the ratio 1:3. There have been limited studies of the acid- or base-catalyzed hydrolysis of a few other cyclic phosphate esters that involve the hemiacetal hydroxyl group of furanoses or pyranoses[8–13], and the acid-catalyzed hydrolysis of aldose 1,2-cyclic phosphates formed by cyclization of aldosyl phosphates has been suggested as a convenient route to aldose 2-phosphates[9].

EXPERIMENTAL

Materials. — Formic acid, acetic acid, and sodium hydrogencarbonate were reagent grade. Imidazole and Tris (free base) were obtained from Sigma and were grade I and reagent grade, respectively. Standard solutions of hydrochloric acid and potassium hydroxide were prepared by using J. T. Baker "Dilut-It" concentrates; water was glass-distilled from deionized feed. The sources of other reagents were as already described[14].

Methods. — Measurements of pH were made using a Radiometer pH 26 pH meter, and GK2321C combination electrode that was standardized against Fisher standard buffers. Absorbance measurements for Glc-1-P or Pi determinations were made with a Unicam SP 1700A spectrophotometer.

Preparation of α-D-glucopyranose 1,2-cyclic phosphate. — The barium salt was prepared by the method of Zmudzka and Shugar[13]; the potassium salt obtained by ion exchange was purified further with acid-washed charcoal as already described[14], and lyophilized. An effective molecular weight was determined by total hydrolysis (1.0M hydrochloric acid, 3 h, 100°) and determination of Pi by the method of Ames[15], and indicated in the various preparations 1.7–4.6 molecules of H_2O per molecule of Glc-1-P in which the phosphoryl oxygen atoms would be ^{18}O-enriched. This ^{18}O-

TABLE I

pH VALUES OF BUFFERS USED FOR HYDROLYSIS OF Glc-1,2-P

Buffer	*pH[a] at 23°*	*pH[b] at 80°*
Hydrochloric acid (0.1M)	0.99	1.09
Hydrochloric acid (0.01M)	2.02	2.07
Formate	3.01	3.08
Formate	4.01	4.07
Acetate	4.99	5.05
Imidazole	6.01	5.08
Imidazole	7.01	6.17
Tris	7.98	6.77
Tris	9.02	7.84
Carbonate	10.02	9.62
	11.03	10.09
Potassium hydroxide (0.01M)	11.96	10.48
Potassium hydroxide (0.01M)	13.00	11.62
Potassium hydroxide (1.0M)	13.97	12.43

[a]Relative to Fisher standard buffer, pH 7.41 at 25°. [b]Relative to Fisher standard buffers, pH 4.16 and 8.88 at 80°.

of cyclic ester. Based on the coupled assay procedure (see later), any Glc-1-P contaminant was less than 1%.

Buffers. — Formate, acetate, and carbonate buffers (0.1M) were prepared by adjustment of the pH of solutions of formic or acetic acid, or sodium hydrogencarbonate with 4M potassium hydroxide. Imidazole and "Tris" buffers (0.1M) were prepared by adjustment of the pH of solutions of imidazole or Tris (free bases) with concentrated hydrochloric acid. The pH values of these solutions at 23 and 80°, are indicated in Table I.

Hydrolysis of α-D-glucopyranose 1,2-cyclic phosphate. — In the case of hydrolyses carried out at pH $\geqslant$ 2, 50.0 ml of buffer was pipetted into a 125-ml Erlenmeyer flask or 250-ml Nalgene bottle (the latter was necessary at higher pH values to prevent silicate contamination, which interferes with Pi assays) and the buffer equilibrated in a constant-temperature bath for 20 min. Solid Glc-1,2-P, enough for a final concentration of about 2.5mM, was then added and the flask or bottle was shaken and returned to the bath. At various times, 5-ml aliquots of solution were transferred to cold test-tubes in an ice bath. These "quenched" samples were kept at 4° or lower until analyzed (as described later). For reactions in 0.1M hydrochloric acid, where the hydrolysis is faster and where the acid concentration is sufficiently high to affect the Pi assay, the procedure was modified so that the concentration of the Glc-1,2-P was increased to 27.5mM and the reaction was quenched by pipetting a 1.0-ml aliquot of the solution into 10.0 ml of ice-cold 0.1M acetate buffer, pH 4.78. These quenched samples were stored at 4° or lower until assayed for Glc-1-P, Pi, and total phosphate by the following methods.

Determination of α-D-glucopyranosyl phosphate. — The concentration of Glc-1-P was determined by using the coupled assay with phosphoglucomutase plus D-glucose 6-phosphate dehydrogenase. Each solution was assayed in duplicate as described earlier[14], except that the concentration of $NADP^+$ was doubled. The quenched reaction solutions were mixed with the assay solution in either the ratios 100 μl:2.9 ml or 50 μl:1.0 ml, and the increases in absorbance at 340 nm resulting from reduction of $NADP^+$ were converted into Glc-1-P concentrations by using 6.22×10^3 $M^{-1}cm^{-1}$ as the extinction coefficient of NADPH at 340 nm. A standard curve prepared by using solutions of Sigma $Na_2Glc\text{-}1\text{-}P \cdot 3.5\ H_2O$ was linear over a range of absorbance changes of 0–1.0 unit, and yielded ε^{340} (NADPH) $= 6.1 \times 10^3$ $M^{-1}cm^{-1}$. Standards of Glc-1-P were also used at various other times to ensure that the assay was functioning properly.

Determination of inorganic phosphate. — The concentration of Pi in the hydrolyzates was determined in duplicate by the Lowry–Lopez method[16], which does not hydrolyze even such sensitive phosphate esters as Glc-1-P. Controls showed that, under the conditions of this assay, there was no measurable release of Pi from Glc-1,2-P. In most instances 1.0 ml of reaction mixture was added directly to 3.0 ml of Lowry–Lopez reagent and the mixture incubated for 5–10 min at 30°. The exact procedure was checked by appropriate standard curves. A blank (water) and a standard of Pi were always run concurrently. Separate control-experiments showed that blanks made from 0.1 or 0.01M hydrochloric acid, any of the buffers, or 0.01–1.0M potassium hydroxide, gave the same A^{700} value as water. In some cases, the mixtures were sufficiently acidic or basic or sufficiently buffered that the A^{700} readings with Pi present were affected, however. Thus, with pH 10 carbonate buffer and with 0.1M potassium hydroxide, readings lower than normal resulted, and with pH 11 carbonate buffer or with M potassium hydroxide this method of analysis was not useful.

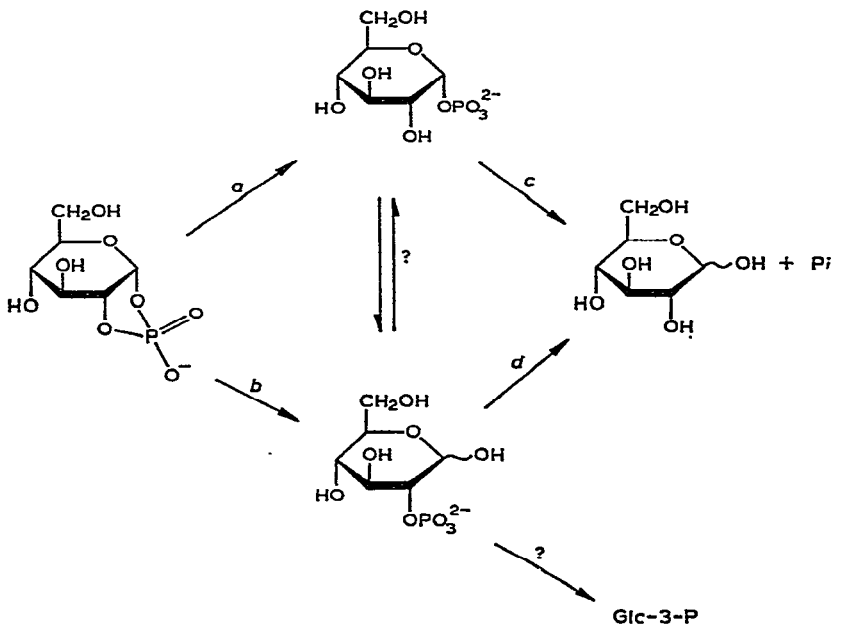
Analyses for total phosphate. — In order to determine the total phosphate content of a solution, a mixture of 1.0 ml of reaction mixture and 9.0 ml of M hydrochloric acid was incubated for 3 h at 100°. After cooling, a 1.0-ml aliquot was added to 3.0 ml of Ames reagent[15], and the mixture was incubated for 1 h at 40°. A standard of inorganic phosphate and a blank were incubated concurrently. It should be noted that M hydrochloric acid and water blanks give the same absorbance readings, but when the standard Pi was in M hydrochloric acid, the final absorbance was about 10% lower than when the standard was in water. Values used are based on the standard in M hydrochloric acid.

Hydrolyses of α-D-glucopyranose 1,2-cyclic phosphate, in ^{18}O-enriched water. — For hydrolysis in 0.1M sodium hydroxide, the solution was made up of 80 mg (260 μmol) of Glc-1,2-P barium salt, 3 ml of 1.6 atom% ^{18}O-enriched water, and 0.1 ml of 3M sodium hydroxide. The sample was heated for 5 min in a boiling-water bath, and then cooled in cold water and neutralized with M hydrochloric acid. This solution was passed through a column of 3 ml of Dowex-50 (H^+), and the effluent was adjusted to pH 8.5 with concentrated ammonium hydroxide. The Glc-1-P and Glc-2-P were separated by chromatography on a column (1.5 × 18 cm) of Dowex-1 (Cl^-), eluted

with 1 l of 0.025M ammonium chloride, 0.01M $Na_2B_4O_7 \cdot 10H_2O$ (pH adjusted to 8.3 with hydrochloric acid), and then 1 l of 0.025M ammonium chloride (pH adjusted to 8.3 with ammonium hydroxide). These conditions are based on those used by Khym and Cohn[17] for the separation of Glc-1-P and D-glucose 6-phosphate; the Glc-2-P was eluted from the column shortly after the second eluent was started, and was shown to be free of Glc-1-P by use of the coupled assay (compare Determination of Glc-1-P). The Glc-2-P was hydrolyzed with alkaline phosphatase and the Pi obtained was analyzed for ^{18}O-content as described earlier[2] for analyses of Glc-1-P. The procedure used for hydrolysis in 0.08M hydrochloric acid was as already described, except that 0.02 ml of concentrated hydrochloric acid (about 11.7M) was used per 3 ml of ^{18}O-enriched water, and the hydrolysis time was 4 min. The Glc-2-P was purified and analyzed as already described.

RESULTS

Effects of pH and temperature on the rate and products of hydrolysis of Glc-1,2-P. — The hydrolysis of Glc-1,2-P can give rise initially to either of the monoesters Glc-1-P or Glc-2-P, and each of these products may undergo further hydrolysis to D-glucose and Pi. In acid solutions, the monoesters might also isomerize, as glycerol phosphate, for example, is known to do[18]. Farrar[19] has suggested that the acid-catalyzed hydrolysis of Glc-2-P may involve an isomerization to the more-labile Glc-1-P. We are particularly interested in knowing the effect of temperature and pH on the relative rates of steps *a* and *b*, and also of *a* and *c*, as the ultimate yield of



Scheme 1 All structures are shown arbitrarily in fully dissociated forms

Glc-1-P depends on both of these.

Hydrolyses in 0.1M or 0.01M hydrochloric acid. — The results of hydrolyses of Glc-1,2-P in 0.1M hydrochloric acid at various temperatures are summarized in Fig. 1. At 23.5 and 40°, hydrolysis of the cyclic ester is complete within about 30 min and leads to 15–18% of Glc-1-P, which is stable under these conditions for at least one h. However, at 60°, hydrolysis of Glc-1-P becomes apparent (that is, Pi is formed) before hydrolysis of the cyclic ester is complete (that is, Glc-1-P is still increasing). At 80°, the hydrolysis of Glc-1-P is sufficiently fast relative to its rate of formation that the maximum concentration of Glc-1-P (10%) is only about half of the Pi concentration when the latter reaches a plateau after 20–60 min. After 20 h at 80°, formation of Pi reaches 100%. At 100°, step *a* seems to be at least largely rate-limiting in the sequence Glc-1,2-P → Glc-1-P → D-glucose + Pi, as the concentration of Glc-1-P was less than 1% at the points where it was determined. Data of Bunton *et al.*[4], and our independent measurements of the rate of hydrolysis of Glc-1-P under some of these conditions are consistent with the foregoing interpretation. Therefore, in 0.1M hydrochloric acid, hydrolysis of Glc-1-P has a greater E_a (energy of activation) than does reaction *a*, whereas the E_a values of the paths *a* and *b* must be nearly identical.

In 0.01M hydrochloric acid, we have not done as complete a study at short reaction-times, but the situation is similar to that in more-concentrated acid (see Fig. 2). For example, at 80° the ratio of rates of steps *a*:*b* is about 1:3, based on the plateau in release of Pi at 60–90 min, but hydrolysis of Glc-1-P is significant before

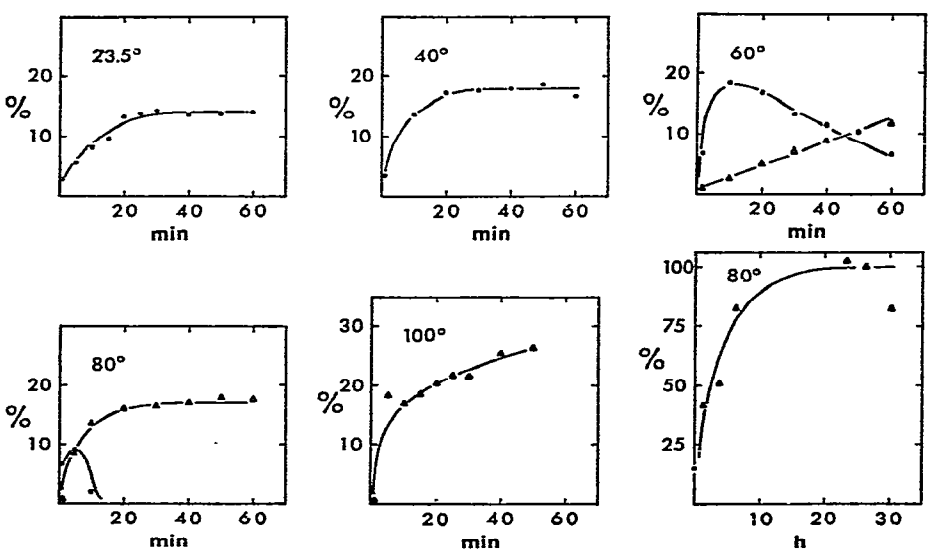


Fig. 1. Progress curves for hydrolysis of Glc-1,2-P in 0.1M hydrochloric acid at various temperatures. The formation of Glc-1-P (●) and Pi (▲) is indicated as a percentage of total phosphate. (Concentrations < 1% not usually plotted.)

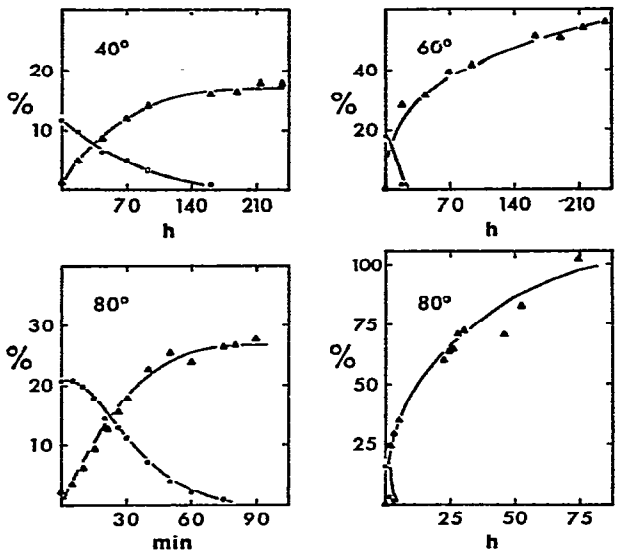


Fig. 2. Progress curves for hydrolysis of Glc-1,2-P in 0.01M hydrochloric acid at various temperatures. The formation of Glc-1-P (●) and Pi (▲) is indicated as a percentage of total phosphate. (Concentrations < 1% were not usually plotted.)

hydrolysis of the cyclic ester is complete; thus the concentration of Glc-1-P reaches 20% within 1 min but liberation of Pi is also evident.

In 0.1M formate buffer, pH 3 or 4, the rates of steps *a* and *c* are comparable and low. Thus, at 80°, Glc-1-P reaches a maximum concentration of about 5% after 2 h, and formation of Pi reaches 100% at about 100 h. At 60°, Glc-1-P reaches a peak at about 10% after 30 h, and Pi reaches only 40% after 360 h. At 40°, Glc-1-P is about constant over the period 80–300 h, and Pi reaches only 10% after 360 h. Therefore, at this pH, lowering the temperature does not spread the rates of reactions *a* and *c* as happens in 0.1M hydrochloric acid. At pH 4 and 80°, the reaction pattern resembles that at pH 3; the concentration of Glc-1-P is constant at 4% up to about 100 h, and the Pi concentration builds up to nearly 80% by 180 h.

In the pH range 5–7 at 80°, the results are complicated, and uninteresting from our particular point of view. At hydrolysis times up to 200 h the concentration of Glc-1-P never exceeds about 10%, and release of Pi also occurs. In some cases, both the Glc-1-P and Pi concentrations appeared to level-off at non-zero values.

At pH values $\geqslant$ 8, the hydrolysis behavior is quite simple: Glc-1-P builds up to about 25% and is stable for periods of time that are long with respect to those required for ring opening of the cyclic ester. Formation of Pi is also essentially nil, and thus Glc-2-P is also stable. Some examples of results at these pH values are given in Fig. 3. Also, in Table II, the approximate times required for the concentration of Glc-1-P to reach one-half of the final level are given. One minor exception to this behavior is observed at pH 13, where, at times very much longer than those needed

TABLE II

APPROXIMATE HALF-TIMES FOR FORMATION OF Glc-1-P FROM Glc-1,2-P AT 80°

Buffer	*pH*	*Half-time*
Tris (0.1M)	7.84	>100 h
Carbonate (0.1M)	9.62	~1 h
Potassium hydroxide (0.01M)	10.48	~7 min
Potassium hydroxide (1M)	12.43	complete by 2.5 min

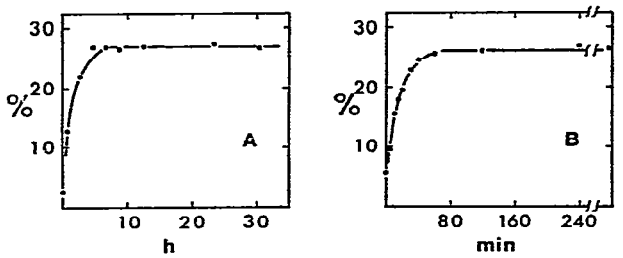
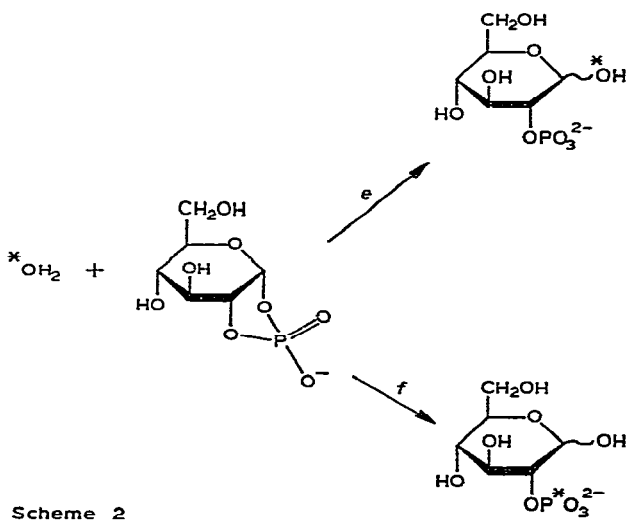


Fig. 3. Progress curves for hydrolyses of Glc-1,2-P at 80° in (*A*) 0.1M carbonate, pH 9.62, or (*B*) 0.01M potassium hydroxide. The formation of Glc-1-P is indicated as a percentage of total phosphate. The Pi concentration is not indicated, as it was remained constant at about 1%. (The last point in part *B* is at 405 min.)

for ring opening, the concentration of Glc-1-P decreases apparently linearly, from 27% after 1 h to 24% after 50 h, but the Pi concentration remains zero.

Position of ring cleavage in hydrolysis of Glc-1,2-P to yield Glc-2-P. — Potentially, the hydrolysis of Glc-1,2-P to Glc-2-P could proceed via C–O (path *e*) or P–O (path *f*) cleavage:



Scheme 2

On the basis of hydrolyses of ethylene phosphate as models, both acid and base-catalyzed hydrolyses would be expected[20] to occur via pathway *f*. However, if hydrolyses of Glc-1-P are taken as models, then pathway *e* would be expected[4] in acid. The alternatives are distinguishable by carrying out the hydrolyses in ^{18}O-enriched water, and examining the Glc-2-P for ^{18}O enrichment in the phosphoryl-oxygen positions. As shown in Scheme 2, hydrolysis of Glc-1,2-P to Glc-2-P via C–O cleavage (path *e*) gives a phosphate group, and hence Pi, of normal isotopic composition, but P–O cleavage (path *f*) leads to enrichment of the phosphate group, and hence of Pi.

We have hydrolyzed Glc-1,2-P in ^{18}O-enriched water solutions of 0.1M sodium hydroxide and 0.08M hydrochloric acid. The Glc-2-P was recovered by ion-exchange chromatography and hydrolyzed further in ordinary water to D-glucose and Pi by using alkaline phosphatase as a catalyst. Under these conditions, the hydrolysis of Glc-2-P proceeds by P–O cleavage, and so the Pi formed contains 3 oxygen atoms derived from the Glc-2-P and one from solvent. The isotopic content of the Pi formed was then examined by mass-spectral methods, as already described[2].

The results of these experiments are given in Table III. They indicate about 1.0 oxygen atoms of solvent introduced into the phosphate group of Glc-2-P during hydrolysis in 0.1M sodium hydroxide, and about 1.5 oxygen atoms of solvent introduced during hydrolysis in 0.08M hydrochloric acid. Based on our other ^{18}O-analyses[2,14] our experimental error (in terms of a standard deviation) is about 11%, and so that the differences between the individual acid- or base-hydrolysis experiments are not significant.

DISCUSSION

As already noted, Zmudzka and Shugar[1] found that crude extracts of a variety

TABLE III

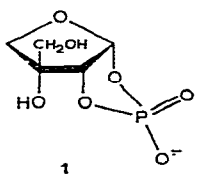
^{18}O-ENRICHMENT OF INORGANIC PHOSPHATE OBTAINED BY ALKALINE PHOSPHATASE-CATALYZED HYDROLYSIS OF Glc-2-P[a]

Sample of Glc-2-P from	*Atom % Excess ^{18}O*	*^{18}O per Glc-2-P (mol)*
Hydrolysis of Glc-1,2-P in 0.08M hydrochloric acid[b]	0.631	
	0.597	
	0.577	
	Av. 0.602 ± 0.027	1.5
Hydrolysis of Glc-1,2-P in 0.1M sodium hydroxide[b]	0.430	
	0.400	
	0.358	
	Av. 0.396 ± 0.036	1.0

[a]The Glc-2-P was the product of hydrolysis of Glc-1,2-P in water with "1.595" atom% excess oxygen-18. [b]4 min at 100°. [c]5 min at 100°.

of tissues catalyze the hydrolysis of Glc-1,2-P and yield Glc-1-P as the exclusive product, but we have not found conditions for chemical hydrolysis where the yield of Glc-1-P exceeds about 25%. Paladini and Leloir[7] earlier reported that hydrolysis of Glc-1,2-P in 0.05M sulfuric acid at 100° yields D-glucose and Glc-2-P in the ratio 1:3, and hydrolysis in 0.1M sodium hydroxide yields Glc-1-P and Glc-2-P also in the ratio 1:3. However, we have found that hydrolysis of Glc-1,2-P in 0.1M hydrochloric acid at 23.5 or 40° gives Glc-1-P and Glc-2-P. Their data and ours are consistent with Farrar's report that, at 100°, Glc-2-P is hydrolyzed in 0.05M sulfuric acid with a half-time of 137 min and in 0.1M sodium hydroxide with a half-time of 97 min. The hydrolyses of other five-membered, cyclic phosphate diesters (involving the hydroxyl group on the anomeric carbon atom of a monosaccharide) also yields, as the major product, the monoesters in which there is a free, hemiacetalic hydroxyl group[9,10,12,13].

We can compare the rate of hydrolysis of Glc-1,2-P with the simplest five-membered cyclic ester, ethylene phosphate, which is hydrolyzed in 0.10M perchloric acid at 30° with a half-time[21] of 5.6 min. In 0.1M hydrochloric acid at 40°, we observe a half-time of about 10 min for hydrolysis of Glc-1,2-P. Our results and those for ethylene phosphate may also be compared with those for the five-membered cyclic phosphates α-D-apio-D-furanosyl 1,2-cyclic phosphate (**1**) and α-D-apio-L-furanosyl 1,2-cyclic phosphate (**2**), whose hydrolyses have been studied by Mendicino and Hanna[11]. In 0.5M sulfuric acid at 26°, these esters have half-times for hydrolysis of about 2 h. Therefore according to these data, fusion of the five-membered cyclic ester to a second five-membered ring causes a considerable diminution of rate.



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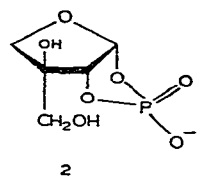

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However, ribonucleoside cyclic 2′,3′-monophosphates, which also contained fused five-membered rings, are hydrolyzed about as rapidly as ethylene phosphate. For example, in 0.1M hydrochloric acid at 18° hydrolysis of cytidine cyclic 2′,3′-monophosphate is complete[22] in 1 h. (Gerlt *et al.*[23] have discussed the effects of other types of ring fusion on the thermodynamics and kinetics of hydrolysis of cyclic phosphate diesters.)

The fact that the hydrolysis of Glc-1,2-P yields as much Glc-1-P at low pH as is formed at high pH suggests that, even in acid, the rate of attack of water at the phosphorus center is at least comparable to that for direct C–O bond cleavage to form Glc-2-P. We have attempted to demonstrate the position of opening of the phosphate diester ring when Glc-1,2-P is hydrolyzed to Glc-2-P, by conducting hydrolyses in ^{18}O-enriched water. The results of these experiments are given in Table III. The particular conditions used (namely, unlabelled Glc-1,2-P and ^{18}O-enriched water) were chosen because they were convenient and because C–O bond-

cleavage would be readily recognized by a lack of incorporation of ^{18}O into Glc-2-P.

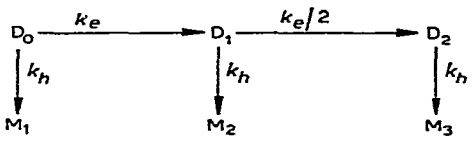
Our result of 1.0 atom of ^{18}O per Glc-2-P formed by hydrolysis of Glc-1,2-P in 0.1M sodium hydroxide at 100° is consistent with a mechanism of P–O bond cleavage. The basic hydrolysis (at 25°) of ethylene phosphate has been shown to occur via P–O cleavage and without significant exchange of reactants or products[20].

The incorporation of 1.5 atoms of ^{18}O per Glc-2-P formed by hydrolysis in 0.08M hydrochloric acid cannot be given a unique interpretation, but the most likely one is that Glc-2-P is formed via P–O cleavage and that Glc-1,2-P undergoes an exchange reaction with water (in the absence of hydrolysis). In the hydrolysis of ethylene phosphate in 0.10M perchloric acid at 25°, Haake and Westheimer[20] found that the cyclic ester exchanged about 20% as fast as it is hydrolyzed, but that exchange of the product (2-hydroxyethyl dihydrogenphosphate) is very much slower. They also found, at 100° in 0.5 and 5.0M perchloric acid, that dimethyl hydrogenphosphate exchanges about 5–10% as fast as it hydrolyzes *by P–O cleavage*, and that methyl dihydrogenphosphate exchanges much more slowly than it hydrolyzes. It is, therefore, unlikely that the incorporation of ^{18}O into the Glc-2-P is a result of exchange with solvent following opening of the phosphate diester ring. If Glc-1,2-P is hydrolyzed by P–O cleavage, then the incorporation of 1.5 atoms of ^{18}O per Glc-2-P requires that the ratio of the rates of exchange and hydrolysis be 0.66 (see Appendix for calculation).

Also with regard to the possibility of C–O cleavage, it is interesting that, at pH 1 at 80 and 100°, the rate of hydrolysis of Glc-1-P is comparable to the rate of ring opening of Glc-1,2-P, and that under these conditions Glc-1-P is hydrolyzed by C–O cleavage[4]. At 26° in 0.50M sulfuric acid, compounds **1** and **2** undergo ring opening (presumably to a mixture of the 1- and 2-phosphates[12]) at rates less than one-tenth of those for hydrolysis of the corresponding 1-monophosphates, which would also be expected to undergo hydrolysis by C–O cleavage[24]. These results suggest that, if any of the cyclic esters hydrolyze in acid by C–O cleavage, then they do so about as fast as (in the case of Glc-1,2-P), or considerably slower than (in the case of **1** and **2**), the corresponding monophosphates hydrolyze by C–O cleavage. Therefore, at least, there is no manifestation of strain in the five-membered phosphate ring in terms of accelerated C–O bond cleavage. Perhaps, there are special effects in the cyclic esters that lead to decreased rates of such C–O bond-cleavages.

APPENDIX

For the exchange and hydrolysis of Glc-1,2-P in ^{18}O-enriched water, the possible reactions are shown in Scheme 3, where D_i is Glc-1,2-P, having i atoms of



Scheme 3

oxygen incorporated from solvent, and M_i is monoester (Glc-1-P + Glc-2-P) having i atoms of oxygen from solvent. Because each step in this scheme is irreversible, the extent of incorporation of ^{18}O into Glc-2-P after complete hydrolysis of the Glc-1,2-P may be readily written. For example, the fraction of Glc-1,2-P that will be converted into M_2 is $[k_e/(k_h + k_e)] \times [k_h/(k_h + 0.5\,k_e)]$. In this way, eq. *1* can be written.

$$\text{Number of } ^{18}\text{O atoms per Glc-2-P} = \frac{M_1 + 2M_2 + 3M_3}{M_1 + M_3 + M_3} = \frac{2 + 3(k_e/k_h)}{2 + (k_e/k_h)} \qquad (1)$$

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