Studies on the Mechanism of Isomerization and Oxygen-18 Exchange of Glycerophosphoric Acid in Aqueous Solutions[1]

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Abstract: The rate of acid-catalyzed isomerization of glycerophosphoric acid has been measured both by periodic acid oxidation and by nmr techniques as a function of acid and substrate concentrations, temperature, and deuterium atom % in solvent water. The observed rates of isomerization are about 10^3 times the rates of hydrolysis of the phosphoglyceric acids. Most of the difference in rates of the two reactions can be accounted for by the difference in apparent activation energies. The rates of isomerization and hydrolysis increase on changing the solvent from H_2O to D_2O, indicating a fast protonation of glycerophosphoric acid prior to the rate-determining step. A kinetic study of O^{18} exchange between water and glycerophosphoric acid indicate that the isomerization occurs both with and without the formation of a cyclic intermediate. A detailed kinetic treatment is given for the dual-path isomerization mechanism.

The isomerization of β-glycerophosphoric acid was discovered by Bailly[3] who showed that in strong acid solutions the isomerization rate is much greater than that of hydrolysis. In the present work, the acid-catalyzed isomerization of glycerophosphoric acid has been chosen for detailed kinetic study both because of the biochemical importance of glycerophosphate itself and as a simplified model for more complex reactions such as the catalytic hydrolysis of ribonucleic acids.[4] While a simple model can only suggest what may be possible in a more complex system, a knowledge of the basic chemistry involved may provide the foundation as well as the guideline for deeper understanding.[5-7]

In this study the rate of isomerization of glycerophosphoric acid was measured by two methods. The first method involved measurements of the integrated nmr spectra of the mixed isomers. In the second method, α-glycerophosphate was oxidized by periodic acid to a labile phosphate ester which was subsequently hydrolyzed and the concentration of the liberated inorganic phosphate determined. For comparison the rate of hydrolysis of glycerophosphoric acid was also determined under the same conditions as a function of hydrogen ion concentration and temperature. To obtain detailed information regarding the isomerization reaction, measurements were also made in D_2O- and H_2O^{18}-enriched aqueous strong acid solutions.

The rate of O^{18} incorporation into the glycerophosphates was also measured for a mixture of α- and β-glycerophosphates at isomerization equilibrium, when the kinetic treatment of O^{18} exchange between glycerophosphates and water is considerably simplified. The O^{18}-incorporation data can be combined with the kinetic isomerization data to demonstrate that the isomerization occurs *via* at least two reaction paths. In the first path, the isomerization proceeds *via* the formation of a cyclic phosphate diester which is subsequently hydrolyzed to give the other isomer with concomitant O^{18} exchange. In the other path, the cyclic intermediate is not formed and hence no O^{18} exchange should occur. A kinetic treatment is developed which quantitatively correlates several kinds of rate data.

Experimental Section

Reagents. β-glycerophosphate, disodium salt pentahydrate, Grade I, and α-glycerophosphate, disodium salt hexahydrate, Grade X, Fiske and Subbarow Reducer were all from Sigma Chemical Co. Deuterium oxide (99.77%) and O^{18}-enriched (1.4%) water were from The Stuart Oxygen Co. Alkaline phosphatase (calf intestine), B grade, was supplied by CalBiochem. All the other chemicals were of reagent grade.

Glycerophosphoric Acid Solutions. Solutions of glycerophosphoric acid (GPA) for isomerization experiments were prepared by dissolving disodium β-glycerophosphate pentahydrate in a known amount of water, cooling in an ice bath, and adding a calculated amount of concentrated acid. Solutions of GPA in sulfuric acid containing 95% D_2O were prepared in the same way by dissolving the glycerophosphate in D_2O and adding the calculated amount of the concentrated acid.

Since commercial concentrated perchloric acid contains 30% H_2O, which cannot be removed by evaporation due to explosion hazard, solutions of GPA in perchloric acid and 99% D_2O were prepared by dissolving the glycerophosphate, in which the exchangeable hydrogen atoms had been replaced by deuterium atoms, in D_2O and adding the calculated amount of $DClO_4$ in D_2O. The $DClO_4$ in D_2O solution was prepared by passing a solution of $NaClO_4$ in D_2O slowly through a cation-exchange column which had been converted to the D^+ form by washing with 99.7% D_2O until the effluent contained less than 1% H_2O as determined from the nuclear magnetic resonance (nmr) spectrum.

The exchangeable hydrogen atoms in disodium glycerophosphate were replaced with deuterium atoms by repeated evaporation with 99.7% D_2O under reduced pressure. The solid sample was dried over phosphorus pentoxide in a vacuum dessicator (1 mm) for 2 days at room temperature before use.

Kinetic Measurements of Isomerization. Since an equilibrium mixture of GPA at 50–100° was found in the present work to contain about 86% α-GPA and 14% β-GPA, the rate of isomerization was measured by starting from the β form. The sodium salt of β-glycerophosphate used in these measurements contained only 0.1% α-glycerophosphate as an impurity. Both nmr and periodate oxidation analysis were employed to follow the progress of the reaction.

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(2) National Institutes of Health Predoctoral Fellow, 1962–1966.

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For kinetic runs followed by the nmr method, capped, thin-walled glass nmr tubes containing 0.5-ml samples of the reaction solution were kept at a constant temperature in a heat bath. These sample tubes were transferred one by one to an ice bath at regular intervals to quench the reaction and kept there until the nmr spectra were taken in a Varian A-60 nmr spectrometer. The temperature in the nmr probe was kept at 5° by the Varian V-6040 variable-temperature controller. β-GPA in D_2O has a doublet with 1.2-ppm chemical shift relative to water due to splitting of the four methylene hydrogens by the β-hydrogen and/or the phosphate group and a complex multiplet between the water peak and the doublet. α-GPA in D_2O has two unequal doublets due to the four methylene hydrogen atoms. In a mixture of α- and β-GPA, one of the α-GPA doublets is superposed on the β-GPA doublet, but the other α-GPA doublet is farther downfield and can be distinguished from the other peaks in the nmr spectrum of a mixture of isomers. By taking the ratio of the area of the separate α-GPA doublet to the total area of all peaks for both the α and β isomers, the fraction of GPA present in the α form in a mixture of both isomers can be determined.

For kinetic runs followed by the periodate oxidation method, 0.5- or 1.0-ml samples of the reaction mixture were quenched in an ice bath, immediately neutralized with equivalent amounts of base, and diluted to the concentration required for analysis by the periodate method. The concentrations of α- and β-glycerophosphates were determined by a modification of Burmaster's procedure.[8] α-Glycerophosphate can be quantitatively oxidized by periodic acid to glycolic aldehyde phosphate at pH below 3 and room temperature when the migration rate is negligible. The iodate formed and the excess of periodic acid were destroyed with sodium sulfite, and the glycolic aldehyde phosphate produced was hydrolyzed with hot acid in 1 hr. The resulting orthophosphate was then determined colorimetrically by a modification of the method of Fiske and Subbarow,[9] in which the orthophosphate was converted to phosphomolybdic acid in 0.5 *F* H_2SO_4 solution, reduced with 1-amino-2-naphthol-4-sulfonic acid, and then assayed with a Cary Model 11 spectrophotometer. The β-glycerophosphate was not affected by periodic acid and was not appreciably hydrolyzed during the above hydrolysis of the glycolic aldehyde phosphate.

The total glycerophosphate (α and β forms) was determined in a separate sample by reacting with periodic acid in hot acid solution in which the isomerization, oxidation by periodic acid, and the hydrolysis of glycolic aldehyde phosphate formed all took place at the same time to give 100% conversion to orthophosphate. The amount of β-glycerophosphate was then obtained by subtracting the previously determined value for α-glycerophosphate from the total phosphate.

Kinetic Measurements of Hydrolysis. The rate of acid-catalyzed hydrolysis of glycerophosphoric acid was determined under similar conditions to those used for the isomerization of the phosphoryl group, except that longer periods at high temperatures were required. At successive time intervals, measured aliquots, usually 0.05 to 0.1 ml, of the reaction mixture were taken with a pipet and transferred to volumetric flasks. These samples were diluted and analyzed for inorganic phosphate by the procedure described above.

Synthesis of Glycerol Cyclic Phosphate. The barium salt of glycerol 1,2-cyclic phosphate was synthesized by dehydrating glycerolphosphoric acid with dicyclohexylcarbodiimide (DCC) by a modification of the method of Ukita and co-workers.[10] Disodium glycerophosphate (5 g) was dissolved in 100 ml of water and converted to the free-acid form (GPA) by ion exchange on an Amberlite IR-120 column. The eluate was lyophilyzed and the residue redissolved in a liquid mixture of 50 ml of acetonitrile + 5 ml of pyridine + 50 ml of dioxane + 9.5 ml of tri-*n*-butylamine. DCC (6.0 g) was dissolved in this solution, and the mixture was left standing for 2 days at room temperature. Then 30 ml of water was added to react with the excess DCC, and the precipitated dicyclohexylurea was removed by filtration. By adjusting the "pH" of the filtrate to 9 with aqueous $Ba(OH)_2$ solution, the insoluble barium glycerophosphate was precipitated and centrifuged off. The supernatant was saturated with CO_2 to precipitate the excess $Ba(OH)_2$ as $BaCO_3$ which was removed by centrifugation. After removing the organic solvents, the solution was lyophilized to give a glassy viscous liquid. This glassy liquid was redissolved in water, adjusted to pH 9 with $Ba(OH)_2$, repeatedly extracted with ether, again saturated with CO_2 and filtered to remove the excess $Ba(OH)_2$, and lyophilized to give a white powder of barium glycerol cyclic phosphate which was further purified by recrystallization from aqueous ethanol and dried in a desiccator over Drierite.

The purity of the barium glycerol cyclic phosphate was checked by ascending chromatography on Whatman No. 1 filter paper. The organic phosphates were detected by spraying the chromatograms with Hanes–Isherwood reagent, drying for 1 hr in a cool air stream, and exposing the dried chromatogram to ultraviolet radiation for 2 min. The phosphates appear as blue spots.[11,12] Two different solvent systems were used: (1) 2-propanol–H_2O (1:1); (2) 2-propanol–5 *N* NH_3 solution (aq) (2:1). The first solvent system is convenient to check the barium glycerol 1,2-cyclic phosphate for possible contamination by barium glycerophosphate, since the latter does not travel on paper with this solvent.[13] It was found that no phosphate remained at the origin of the above chromatogram. In the second solvent system, the observed R_f values were 0.64 for barium glycerol cyclic phosphate and 0.26 for disodium glycerophosphate. The literature values for the second solvent system were 0.6 and 0.25–0.35, respectively.[10,13] Although both systems could detect an impurity of less than 2% noncyclic glycerophosphate, none was detected in the barium glycerol 1,2-cyclic phosphate prepared by the above procedure.

Hydrolysis of Glycerol 1,2-Cyclic Phosphate. At room temperatures, glycerol 1,2-cyclic phosphate is not hydrolyzed for at least 24 hr if the pH is between 3.5 and 9.5.[10] For the measurement of hydrolysis, the barium glycerol cyclic phosphate was dissolved in cold water, brought up to the temperature for the reaction, and mixed with the calculated amount of perchloric acid to start the hydrolysis. After the reaction, the solution was neutralized by adding sodium hydroxide and analyzed for α-glycerophosphate as well as the total GPA by the periodic acid oxidation method described above

Kinetic Measurements of Oxygen-18 Exchange. In the preliminary experiments, disodium β-glycerophosphate pentahydrate was dissolved in water containing 1.36 atoms % O^{18}, mixed with the required amount of perchloric acid in an ice bath, and brought up to the temperature for the isomerization and O^{18} exchange to take place simultaneously. For the simplicity of interpretation, most of the O^{18}-exchange rate measurements were carried out at isomerization equilibrium by dissolving the equilibrium amounts (86% α-GPA and 14% β-GPA at 60°) of α- and β-glycerophosphates in O^{18}-enriched aqueous perchloric acid solution and keeping the reaction mixture in glass-stoppered flasks immersed in a heated constant-temperature bath. At predetermined intervals, the reaction was quenched by placing one of the flasks in an ice bath and immediately neutralizing the solution with 10 *N* KOH (aq). Each of the solutions was filtered to remove the precipitated $KClO_4$ and the O^{18} content of the phosphoryl group in glycerophosphate was determined by a modification of the procedure of Cohn.[14] The above filtrate (0.6 ml) was mixed with 8 ml of 0.1 *F* NH_3–NH_4Cl buffer, pH 9.3, 1 ml of 0.6 *F* $MgCl_2$, and 3 mg of alkaline phosphatase. After incubation for 2 hr at 37°, the mixture was transferred to a refrigerator at 0° and left there overnight to complete the precipitation of $MgNH_4PO_4$. The white precipitate was redissolved in 5% trichloroacetic acid and centrifuged to remove the denatured enzyme. The Mg^{2+} in the supernatant was removed by an Amberlite IR-120 column in the H^+ form. The eluate was concentrated by vacuum evaporation and adjusted to pH 4.4 with KOH (aq). The phosphate was reprecipitated as KH_2PO_4 by adding absolute ethanol, washed, and dried *in vacuo* for 16 hr at 100°.

Very little hydrolysis of GPA took place at the temperature and acidity of the above-described O^{18}-exchange experiments. As a check, the above procedure was repeated without alkaline phosphatase. No precipitate of $MgNH_4PO_4$ was formed.

The oxygen of KH_2PO_4 was converted to CO_2 by heating with dry guanidine hydrochloride according to the method of Boyer[15] and analyzed in either a Consolidated Engineering 21-401 mass

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(11) R. S. Bandurski, *J. Biol. Chem.*, **193**, 405 (1951).
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(13) T. Ukita, N. A. Bates, and H. E. Carter, *J. Biol. Chem.*, **216**, 867 (1955).
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(15) P. D. Boyer, D. J. Graves, C. H. Suelter, and M. E. Dempsey, *Anal. Chem.*, **33**, 1906 (1961).

spectrometer or a Nuclide mass spectrometer by the isotope ratio method. The O^{18} content of enriched water was determined by the same procedure as that used for KH_2PO_4, except that it is necessary to heat the sealed tube containing water and guanidine hydrochloride for 2 min for satisfactory yields of CO_2.

The experimentally determined values of O^{18} atom% in CO_2 are equal to the corresponding values of O^{18} atom % in the KH_2PO_4 obtained by enzymatic hydrolysis of glycerophosphate. In order to use these data in the rate equations for O^{18} exchange, it is necessary to make two corrections. First, it is necessary to subtract the excess of O^{18} abundance introduced into the phosphate group during enzymatic hydrolysis of the glycerophosphate in 10 ml of buffer solution containing 0.6 ml of the neutralized reaction mixture in O^{18}-enriched solution. The amount of this O^{18}-enrichment correction was determined from the O^{18} content of the phosphate from an unheated sample of the reaction solution. The only O^{18} incorporation which could occur in this blank experiment is that during enzymatic hydrolysis. Secondly, it is also necessary to multiply the excess O^{18} concentration found in the KH_2PO_4 by four-thirds, since only three of the oxygen atoms were from GPA,; the fourth oxygen atom was introduced into the phosphate group during the hydrolysis by alkaline phosphatase.

Results

Acid-Catalyzed Isomerization. Consider the isomerization reaction

$$\alpha \underset{k_\beta}{\overset{k_\alpha}{\rightleftharpoons}} \beta$$

where for simplicity of notation α and β represent either the isomeric molecular species α-GPA and β-GPA or their molar concentrations, k_α and k_β the corresponding first-order rate constants. The net isomerization rate is

$$-(d\alpha/dt) = k_\alpha{}^\alpha - k_\beta{}^\beta = (k_\alpha + k_\beta)\alpha - k_\beta a \qquad (1)$$

where $a = \alpha + \beta$ is the total molar concentration of GPA. Integration of (1) gives

$$\ln \frac{\alpha_0 - ba}{\alpha - ba} = (k_\alpha + k_\beta)t \qquad (2)$$

where α_0 denotes the initial concentration of α-GPA and $b = k_\beta/(k_\alpha + k_\beta)$. At isomerization equilibrium the concentrations α_∞ and β_∞ satisfy the requirement $k_\alpha\alpha_\infty = k_\beta\beta_\infty$; hence

$$ba = \frac{(\alpha_\infty + \beta_\infty)k_\beta}{k_\alpha + k_\beta} = \frac{\alpha_\infty(k_\beta + k_\alpha)}{k_\alpha + k_\beta} = \alpha_\infty$$

and (2) becomes

$$\ln \frac{\alpha_\infty - \alpha_0}{\alpha_\infty - \alpha} = (k_\alpha + k_\beta)t \qquad (3)$$

Thus $(k_\alpha + k_\beta)$ can be determined from the slope of the linear plot of $\ln [(\alpha_\infty - \alpha_0)/(\alpha_\infty - \alpha)]$ *vs.* t; k_β was then calculated from $\alpha_\infty/\beta_\infty$ and $k_\alpha + k_\beta$. All plots of this kind were found in the present work to be strictly linear. A typical example is given in Figure 1. The experimentally determined values of the apparent first-order rate constants for phosphoryl group migration of 0.5 M β-GPA in various acid solutions in H_2O and D_2O at different temperatures are summarized in Table I. The values of the apparent activation energy E_a and the frequency factor k_0 are calculated from the slopes of the linear plots of log k_β *vs.* $1/T$, assuming that $k_\beta = k_0 e^{-E_a/(RT)}$, and are also included in Table I.

In order to examine the effect of hydrogen ion concentration on the isomerization rate, a series of 0.5 M GPA solutions of constant ionic strength but increasing perchloric acid concentration ($[HClO_4] + [NaClO_4] = 4.0\ F$) was studied at a chosen temperature. The measured values of $(k_\alpha + k_\beta)$ are also listed in Table I. A plot of these values of log $(k_\alpha + k_\beta)$ *vs.* log $[H^+]$ gives a straight line up to $[HClO_4] = 2.0\ M$ with a slope of 0.9, which indicates that the isomerization reaction is approximately first order with respect to $[H^+]$.

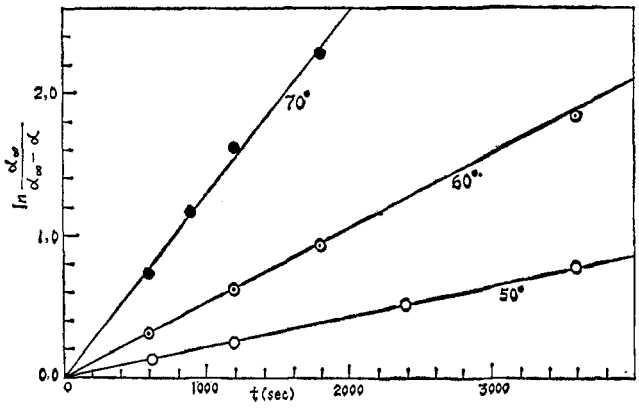


Figure 1. Isomerization of 0.5 M β-glycerophosphoric acid in 1 N $HClO_4$ (aq).

Acid-Catalyzed Hydrolysis of α- and β-GPA. The acid-catalyzed hydrolysis of GPA was also found to follow strictly first-order kinetics. If the reactions are represented by

$$\begin{array}{ccc} \alpha & \underset{k_\beta}{\overset{k_\alpha}{\rightleftharpoons}} & \beta \\ & {\scriptstyle k_\alpha}\searrow \quad \swarrow{\scriptstyle k_\beta} & \\ & P_i + \text{glycerol} & \end{array}$$

where h_α and h_β represent the rate constants for the hydrolysis of α- and β-GPA, respectively, to inorganic phosphate and glycerol, we have

$$dP_i/dt = h_\alpha\alpha + h_\beta\beta \qquad (4)$$

Since the rate of isomerization is much faster than the rate of hydrolysis, the ratio β/α remains at the isomerization equilibrium value throughout the hydrolysis reaction, and consequently we may write the isomerization equilibrium constant K as

$$K = \frac{k_\alpha}{k_\beta} = \frac{\beta}{\alpha} = \frac{a - \alpha - P_i}{\alpha}$$

Table I. Rate of Acid-Catalyzed Isomerization of Glycerophosphoric Acid

Catalyzing acid	Solvent	$(k_\alpha + k_\beta) \times 10^3$, sec^{-1} 2°	50°	60°	70°	80°	100°	E_a for k_β, kcal/mole	k_0, sec^{-1}
1.85 N H_2SO_4	H_2O			0.29		1.38	5.4	18.2	2×10^8
1.85 N D_2SO_4	95% D_2O			0.405		1.99	8.2	18.7	8×10^8
1.4 N D_2SO_4	95% D_2O			0.31	0.685	1.46	4.94	17.5	9.1×10^7
1 N $HClO_4$	H_2O	0.00075	0.22	0.53	1.3			19.7	3.8×10^9
1 N $DClO_4$	99% D_2O	0.0017		0.925				19.2	3×10^9
1 N $HClO_4$ + 3 F $NaClO_4$	65% D_2O			1.06					
1.5 N $HClO_4$ + 2.5 F $NaClO_4$	65% D_2O			1.5					
2.0 N $HClO_4$ + 2.0 F $NaClO_4$	65% D_2O			1.9					
2.5 N $HClO_4$ + 1.5 F $NaClO_4$	65% D_2O			2.2					
3.0 N $HClO_4$ + 1.0 F $NaClO_4$	65% D_2O			2.45					

Table II. Kinetic Data for the Hydrolysis of Glycerophosphoric Acid

Catalyzing acid	Solvent	Initial concn of GPA, M	$(h_\alpha + Kh_\beta)/(1 + K) \times 10^6$, sec^{-1} 80°	100°	120°	E_a', kcal/mole	k_0', sec^{-1}
1.85 N H_2SO_4	H_2O	0.5	0.478	2.89	1.69	24.5	6.3×10^8
3.2 N $HClO_4$	65% D_2O	0.4		6.95			
3.2 N $HClO_4$	H_2O	0.4		6.37			
2.2 N $HClO_4$	H_2O	0.4		4.53			

or

$$\alpha = \frac{a - P_i}{1 + K}$$

Substitution in (4) leads to

$$\frac{dP_i}{dt} = \frac{a - P_i}{1 + K}(h_\alpha + Kh_\beta)$$

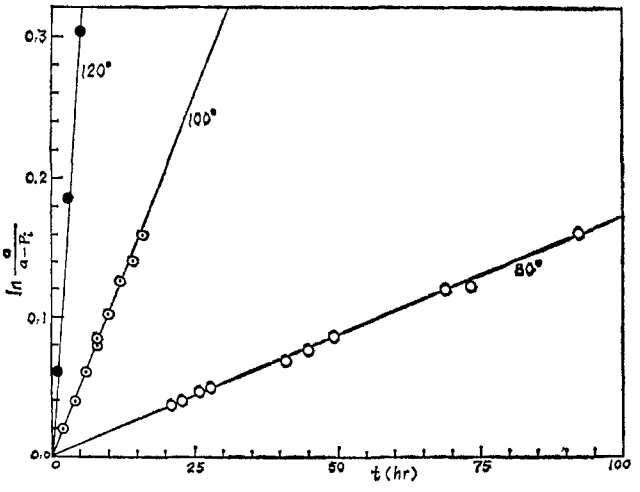


Figure 2. Hydrolysis of 0.5 M glycerophosphoric acid in 1.85 N H_2SO_4 (aq).

which upon integration gives

$$\ln\left(\frac{a}{a - P_i}\right) = \left(\frac{h_\alpha + Kh_\beta}{1 + K}\right)t \qquad (5)$$

The kinetic data for hydrolysis of GPA are summarized in Table II. A typical set of plots according to eq 5 is given in Figure 2.

Since the hydrolysis was followed by determining the concentration of P_i produced and isomerization equilibrium was essentially maintained throughout the hydrolysis reaction, it is not possible to compute h_α and h_β separately from the data in Table II. However, comparison of these values with the data in Table I shows that in general the rate of isomerization of GPA is under the same experimental conditions about 10^3 times faster than the rate of hydrolysis. The apparent activation energy E_a' determined by plotting ln $[(h_\alpha + Kh_\beta)/(1 + K)]$ *vs.* $1/T$ can be used to approximate the activation energy determined from a plot of ln h_β *vs.* $1/T$ if the ratio, of the rate constants h_β/h_α and the equilibrium constant $K = k_\alpha/k_\beta$ are independent of temperature. The approximation is supported by the observation that K is nearly independent of temperature.

At 100°, a factor of 10^3 in the rate of isomerization and hydrolysis reactions corresponds to a difference of 5.1 kcal/mole in activation energy if the two k_0's are equal. Data in Tables I and II show that in 1.85 N H_2SO_4 solution at 100°, the apparent activation energy of hydrolysis exceeds that for isomerization by 6.3 kcal/mole, which indicates that the difference in rates is mainly due to the difference in activation energies.

Table III. Hydrogn-Isotope Effect on Acid-Catalyzed GPA Reactions

Reaction	Initial concn of GPA, M	Catalyzing acid	% D_2O in the solvent D_2O solutions	k^D/k^H 2°	60°	80°	100°
Isomerization	0.5	1.0 N $HClO_4$	99% D_2O	2.1	1.75		
Isomerization	0.5	1.85 N H_2SO_4	95% D_2O		1.4	1.44	1.5
Isomerization	0.5	3.0 N $HClO_4$	65% D_2O		1.15		
Hydrolysis	0.4	3.2 N $HClO_4$	65% D_2O				1.09

Hydrogen-Isotope Effect. The effect of replacing solvent H_2O with D_2O on the rates of both acid-catalyzed isomerization and hydrolysis of GPA is summarized in Table III, in which k^D/k^H denotes the ratio of the corresponding rate constants in D_2O-enriched and ordinary aqueous solutions, respectively. In all cases studied, the rates are faster in solutions containing D_2O, with the acceleration of rate greater near 100% D_2O.

Acid-Catalyzed Hydrolysis of Glycerol 1,2-Cyclic Phosphate to α- and β-GPA. For the reaction

$$\alpha\text{-GPA} \underset{k_{31}}{\overset{k_{13}}{\rightleftharpoons}} \text{glycerol 1,2-cyclic phosphate} \underset{k_{23}}{\overset{k_{32}}{\rightleftharpoons}} \beta\text{-GPA}$$

the individual rate constants for the hydrolysis of glycerol 1,2-cyclic phosphate are experimentally rather difficult to determine by direct measurement. However, the ratio of rate constants k_{31}/k_{32} can be determined by simple measurements of the ratio of products formed by the hydrolysis of the cyclic ester. If the reaction is started from pure glycerol cyclic phosphate and the time of hydrolysis is short enough so that the isomerization of the product α- and β-GPA is negligible, the following simple relationships hold

$$d\alpha/dt = k_{31}c$$

$$d\beta/dt = k_{32}c$$

where c = concentration of the cyclic ester. Therefore,

$$d\alpha/k_{31} = cdt = d\beta/k_{32}$$

and hence

$$\theta = k_{31}/k_{32} = \alpha/\beta \qquad (6)$$

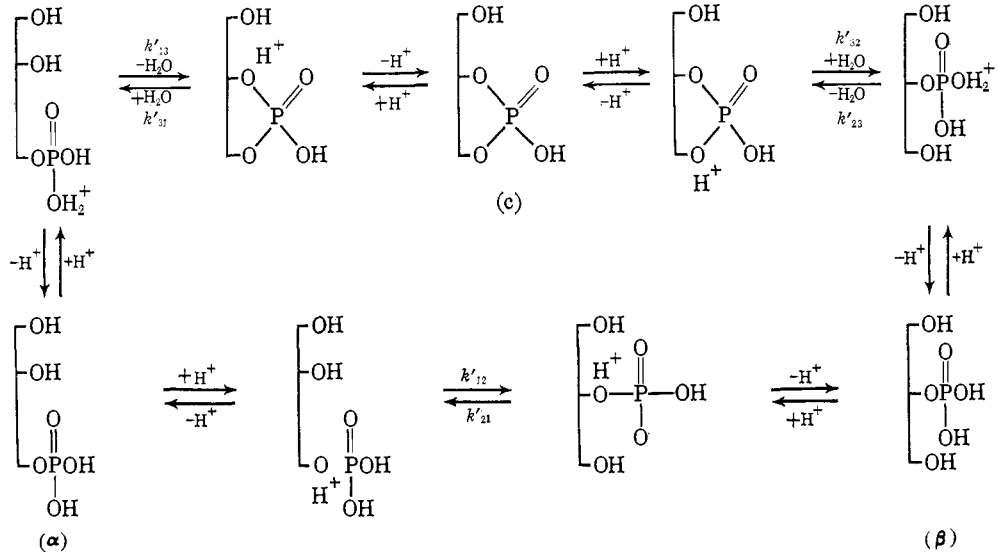
The experimental results are given in Table IV.

Table IV. Hydrolysis of Barium Glycerol 1,2-Cyclic Phosphate in 1 N $HClO_4$ (aq)

Temp, °C	Hydrelysis time, min	α, M	(α + β), M	$\alpha/\beta = k_{31}/k_{32} = \theta$
(Blank)	0	(0.012)	0.495	...
60°	1	0.198	0.495	0.67
23°	5	0.192	0.495	0.64
23°	20	0.198	0.495	0.67
				Av θ = 2/3

Acid-Catalyzed O^{18} Exchange between GPA and Water. Since the isomerization of GPA involves intramolecular phosphoryl group migration, it may proceed *via* the formation of a cyclic phosphate diester (c), followed by hydrolysis, or *via* a concerted mechanism, with direct nucleophilic attack on the phosphorus atom by the oxygen of the neighboring hydroxyl group and simultaneous cleavage of one of the original P–O bonds, or by a combination of both mechanisms. These two pathways for isomerization are shown in Scheme I.

Scheme I. Mechanism of Isomerization and O^{18} Exchange between GPA and Water



Since the rate of hydrolysis of the cyclic phosphodiester to the noncyclic monoester is much faster than the isomerization reactions, the steady-state concentration of the cyclic diester during the isomerization reaction is very low. A study of the rate of O^{18} exchange between GPA and water may provide information on the mechanism of isomerization. If the isomerization of GPA occurs *via* a cyclic phosphodiester intermediate, O^{18} exchange between water and GPA will take place as was reported by Harrison, *et al.*, based on a single experiment.[16] On the other hand, if the isomerization

(16) W. Harrison, P. D. Boyer, and A. Falcone, *J. Biol. Chem.*, 215, 303 (1955).

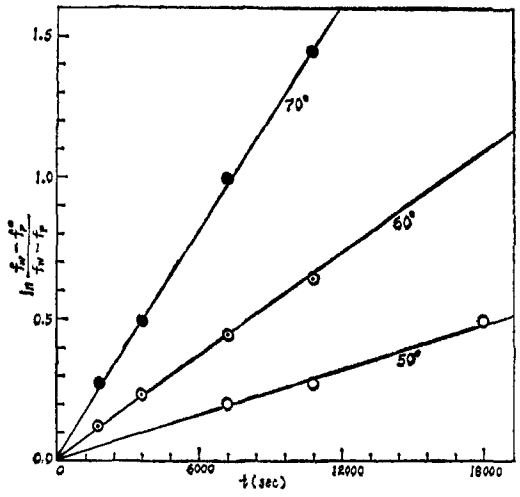
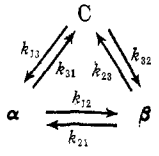


Figure 3. Oxygen-18 exchange between glycerophosphoric acid (0.5 *M*) and water in 1 *N* $HClO_4$ solution.

occurs exclusively *via* a path without involving water as a reactant, no O^{18} exchange will take place.

A kinetic study of the rate of O^{18} exchange was carried out under the same conditions that the rate of over-all isomerization had been measured, but these data were not adequately analyzed because of their complexity. A more thorough study was made on the rate of O^{18} exchange between water and GPA at isomerization equilibrium for which the quantitative treatment of the experimental data is greatly simplified.

For the convenience of kinetic treatment the above reaction pathways may be replaced by the following scheme



where α, β, and c represent α-GPA, β-GPA and the cyclic diester, respectively, and k_{12} and k_{21} are the apparent first-order isomerization rate constants for the lower reaction path without the formation of a cyclic intermediate.

Let f_w = atom-fraction of O^{18} in water, f_p = atom-fraction of O^{18} in the phosphoryl group of GPA, and $a = \alpha + \beta$; then at isomerization equilibrium

$$\frac{\beta}{\alpha} = K = \frac{k_{13}k_{32}}{k_{31}k_{23}} = \frac{k_{12}}{k_{21}}$$

Thus

$$3a(df_p/dt) = (k_{13}\alpha + k_{23}\beta)(f_w - f_p)$$

which gives upon integration

$$3 \ln \frac{f_w - f_p^0}{f_w - f_p} = k_{13}\left(1 + \frac{k_{32}}{k_{31}}\right)\left(\frac{\alpha}{a}\right)t \qquad (7)$$

Since the ratio $k_{31}/k_{32} = \theta$ was already determined from the above-described measurements of the hydrolysis of glycerol 1,2-cyclic phosphate, k_{13} can be calculated from the slope of the linear plot of $\ln [(f_w - f_p^0)/(f_w - f_p)]$ *vs.* *t*. A typical example of such a plot is shown in Figure 3.

Knowing k_{13}, the values of k_{23} can be readily calculated by means of the relationship

$$k_{23} = \frac{k_{13}}{K}\left(\frac{k_{32}}{k_{31}}\right) = \frac{k_{13}}{K}\left(\frac{1}{\theta}\right) \qquad (8)$$

from the experimental values of the over-all equilibrium constant *K*, and θ obtained above. The rate constants k_{13} and k_{23}, obtained from the slope of the linear plots of Figure 3, are listed in Table V.

Table V. Oxygen-18 Exchange Rate Data at Isomerization Equilibrium [0.5 *M* GPA in 1 *N* $HClO_4$ (aq)]

	50°	60°	70°
$k_{13} \times 10^4$, sec^{-1}	0.376	0.825	1.83
$k_{23} \times 10^4$, sec^{-1}	3.2	7.6	18.4

Discussion

The fact that the acid-catalyzed isomerization of GPA is faster in D_2O than in H_2O indicates a pretransition-state fast protonation step as illustrated below.

$$\beta + H_3O^+ \overset{K_{\beta H^+}}{\rightleftharpoons} \beta H^+ + H_2O \text{ (fast equilibrium)}$$

$$\beta H^+ \underset{k_\alpha}{\overset{k_\beta}{\rightleftharpoons}} \alpha H^+ \text{ (rate-determining step)}$$

The rate *R* can be written as

$$R = k_\beta[\beta H^+] = \frac{k_\beta}{K_{\beta H^+}}\beta[H_3O^+] \qquad (9)$$

where $K_{\beta H^+}$ is the ionization constant of the conjugate acid of β-GPA. From (9), it can be seen that the second-order rate constant k^H for the reaction of β-GPA and hydrogen ion is given by $(k_\beta^H/K_{\beta H^+})$, where the superscript H indicates the reaction in water as the solvent. For the same reaction in deuterium oxide as solvent, we have

$$R = \frac{k_\beta^D}{K_{\beta D^+}}\beta[D_3O^+]$$

Therefore the ratio of the rate constants for the two solvents is

$$\frac{k^D}{k^H} = \frac{k_\beta^D}{k_\beta^H}\frac{K_{\beta H^+}}{K_{\beta D^+}} \qquad (10)$$

k_β^D/k_β^H is usually close to unity if there is no kinetic isotope effect at the rate-determining step. For example, if the rate-determining step does not involve proton transfer, the only variation in rate due to the first ratio will be due to general medium effects which are usually small. But since acids are usually weaker in D_2O than in H_2O, the ratio $K_{\beta H^+}/K_{\beta D^+}$ can often be greater than unity, which causes $k^D/k^H > 1$, as shown in Table III.

For the general case where the isomerization takes place through both of the reaction paths discussed above, the rate equations can be formulated in terms of k_{13}, k_{31}, k_{23}, k_{32}, k_{12}, and k_{21} defined in the previous section. Thus we have

$$d\alpha/dt = -(k_{12} + k_{13})\alpha + k_{21}\beta + k_{31}c \qquad (11)$$

$$d\beta/dt = k_{12}\alpha - (k_{21} + k_{23})\beta + k_{32}c \qquad (12)$$

$$dc/dt = k_{13}\alpha + k_{23}\beta - (k_{31} + k_{32})c \qquad (13)$$

At steady state

$$dc/dt = 0; \quad d\alpha/dt = -(d\beta/dt)$$

$$c = \frac{k_{13}\alpha + k_{23}\beta}{k_{31} + k_{32}} \tag{14}$$

Substitution of (14) into (11) gives

$$\frac{d\alpha}{dt} = \frac{k_{31}k_{23}\beta - k_{13}k_{32}\alpha}{k_{31} + k_{32}} - k_{12}\alpha + k_{21}\beta \tag{15}$$

Since

$$K = \frac{k_{13}k_{32}}{k_{31}k_{23}} = \frac{k_{12}}{k_{21}}$$

(15) may be written as

$$\frac{d\alpha}{dt} = \left(\frac{k_{13}}{1+\theta} + k_{12}\right)\left(\frac{a-\alpha}{K} - \alpha\right) \tag{16}$$

where $a = \alpha + \beta + c \approx \alpha + \beta$, and $\theta = k_{31}/k_{32}$. Integration of (16) gives

$$\left(1 + \frac{1}{K}\right)\left(\frac{k_{13}}{1+\theta} + k_{12}\right)t = \ln \frac{a - (1+K)\alpha_0}{a - (1+K)\alpha} = \ln \frac{\alpha_\infty - \alpha_0}{\alpha_\infty - \alpha} \tag{17}$$

Using K determined from equilibrium measurement, θ determined from the hydrolysis of the cyclic phosphodiester, and k_{13} determined from O^{18}-exchange measurements, k_{12} was then calculated from the slope of the linear plots of $\ln [(\alpha_\infty - \alpha_0)/(\alpha_\infty - \alpha)]$ *vs.* t as shown in Figure 1. k_{21} was calculated from K and the so obtained k_{12}.

The rate of isomerization through the cyclic phosphodiester reaction path from α-GPA to β-GPA is

$$R_c = k_{13}\alpha - k_{31}c = k_{32}c - k_{23}\beta \tag{18}$$

which because of (14) becomes

$$R_c = k_{32}\left(\frac{k_{13}\alpha + k_{23}\beta}{k_{31} + k_{32}}\right) - k_{23}\beta = \frac{k_{32}k_{13}\alpha - k_{31}k_{23}\beta}{k_{31} + k_{32}} \tag{19}$$

Meanwhile the rate of isomerization through the direct phosphoryl group transfer path without the formation of the cyclic intermediate is

$$R_d = k_{12}\alpha - k_{21}\beta \tag{20}$$

The rates of isomerization by the two possible reaction paths can be conveniently compared at the initial steady state. For example, when the isomerization is started from pure α-GPA and the initial concentration of β-GPA is approximately zero, we have

$$\frac{R_c}{R_d} = \frac{k_{32}k_{13}}{k_{31} + k_{32}}\frac{1}{k_{12}} = \frac{k_{13}}{k_{12}}\left(\frac{1}{1+\theta}\right) \tag{21}$$

Since $\theta = {}^2/_3$, we have

$$R_c/R_d = ({}^3/_5)(k_{13}/k_{12})$$

The values of k_{13}, k_{12}, k_{23}, k_{21}, K, and R_c/R_d are summarized in Table VI. These values are, within experimental error, consistent with the data in Table I.

Table VI. Rate Constants for the Isomerization and O^{18} Exchange of Glycerophosphoric Acid [0.5 *M* GPA in 1 *N* $HClO_4$]

	50°	60°	70°	E_a, kcal/mole
$k_{13} \times 10^4$, sec^{-1}	0.376	0.825	1.83	17.7
$k_{12} \times 10^4$, sec^{-1}	0.10	0.245	0.59	19.5
$\frac{R_c}{R_d} = \frac{3}{5}\left(\frac{k_{13}}{k_{12}}\right)$	2.26	2.02	1.86	
$k_{23} \times 10^4$, sec^{-1}	3.2	7.6	18.4	
$k_{21} \times 10^4$, sec^{-1}	0.57	1.5	3.94	
K	0.176	0.163	0.15	

The apparent activation energies for the direct isomerization of α-GPA and for the formation of the cyclic glycerol phosphate from α-GPA calculated from the temperature dependence of these rate constants are also listed in Table VI. It may be noticed that the activation energy for the hydrolysis of α-GPA is 6 kcal/mole more than the E_a for isomerization by either reaction path. The data in Table VI show that acid-catalyzed isomerization occurs *via* both reaction paths. Starting from α-GPA as the example, the rate constant k_{13} for the formation of the cyclic intermediate is more than three times the rate constant k_{12} for direct isomerization *via* a concerted mechanism with no O^{18} exchange. But since only a part of the cyclic intermediate formed results in isomerization, the initial rate of net isomerization proceeding *via* the cyclic intermediate is only about twice the rate of direct intramolecular phosphoryl group transfer.