This NMR technique has also allowed us to determine the pK's of α- and β-glucose 6-phosphate as 6.193 ± 0.008 (α) and 6.134 ± 0.005 (β).[10] The pK of β-glucose 6-phosphate is lower as the result of an "anomeric effect" of 1.143 ± 0.001 on the acid dissociation constant. The reason for this is likely that formation of a hydrogen bond between the anomeric hydroxyl (pK more acidic than bulk water by ~2 pH units) and the phosphate group stabilizes deprotonation of the latter in the β anomer, but not in the α anomer where such a hydrogen bond cannot readily form.

Acknowledgment. This work was supported by NIH Grant GM 18938. W.B.K. holds a NIH postdoctoral fellowship (GM09677).

Registry No. ^{18}O, 14797-71-8; phosphate, 14265-44-2; glycerol 3-phosphate, 57-03-4; glucose 6-phosphate, 56-73-5.

(10) The chemical shift of the α anomer is upfield from that of the β anomer by 0.027 ppm when both are deprotonated, although the difference is 0.009 ppm in the opposite direction for the protonated esters (these values come from the fits to eq 3). The difference (α upfield of β) reaches as much as 0.134 ppm near the pK.

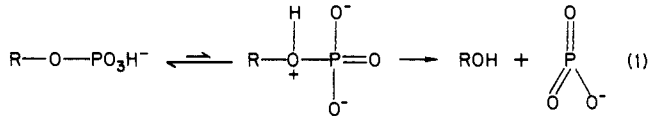
Secondary ^{18}O Isotope Effects on the Hydrolysis of Glucose 6-Phosphate

Paul M. Weiss, W. B. Knight, and W. W. Cleland*

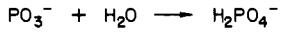
Department of Biochemistry, University of Wisconsin Madison, Wisconsin 53706

Received May 28, 1985

Two limiting mechanisms have been considered for phosphate-transfer reactions. The hydrolysis of monoprotonated phosphate monoesters has been thought to involve a dissociative mechanism with a "metaphosphate" intermediate.[1] This mechanism requires preequilibrium proton transfer to the bridge atom prior to P–O bond cleavage:



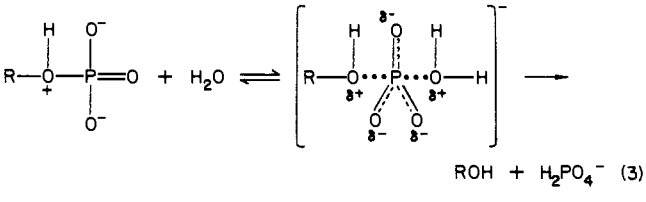
(1)



By contrast, reaction of phosphate triesters is thought to involve an associative reaction with a pentavalent intermediate, especially in cases where the eventual leaving group begins in an equatorial position and pseudorotation must occur to permit it to leave from an axial position:[1]

$$(R\text{-}O)_3P{=}O + OH^- \rightleftharpoons (R\text{-}O)_3P(-O^-)\text{-}OH \rightarrow (R\text{-}O)_2P(=O)\text{-}O^- + ROH \quad (2)$$

Between these limiting models are S_N2 mechanisms with various axial and equatorial bond orders in the transition state, but no free intermediate. For hydrolysis of a monoprotonated phosphate monoester, for example, an alternate formulation to the metaphosphate mechanism would be



(3)

where the positive charge in the transition state might be shared between the two axial oxygens and the phosphorus, depending on the bond orders. Two groups have recently proposed such a mechanism with low axial P–O bond order in place of a metaphosphate mechanism for phosphate transfer from *N*-phosphorylpyridines to pyridines or primary amines.[2,3]

One potential way to distinguish these mechanisms is by measurement of secondary ^{18}O isotope effects resulting from ^{18}O-substitution in the nonbridge oxygens of a phosphate ester. In an ionized monoester these oxygens have a formal bond order of $^4/_3$ to phosphorus, while in metaphosphate the bond order is $^5/_3$, and a pentavalent adduct contains single bonds. Intuition thus predicts an inverse kinetic isotope effect for a metaphosphate mechanism (after correction for the equilibrium ^{18}O isotope effect on the preequilibrium proton transfer to the bridge oxygen), a normal isotope effect for formation of a pentavalent intermediate, and an isotope effect for a S_N2 reaction that depends on the axial and equatorial bond orders in the transition state.[4]

We have measured secondary ^{18}O kinetic isotope effects resulting from ^{18}O substitution in the three nonbridge oxygens on the hydrolysis of glucose 6-phosphate at 100 °C in 50 mM phthalate, pH 4.5. The reaction involves only P–O bond cleavage, has a half-life of 12 h, and is typical of reactions that are thought to proceed by a largely dissociative "metaphosphate" mechanism.[5,6] This reaction was chosen because of the ease of measuring the isotope effects and because we intend to measure similar isotope effects in enzymatic reactions involving glucose 6-phosphate.

^{18}O isotope effects were measured by the remote label method[7] in which [1-^{13}C]glucose 6-[$^{18}O_3$]phosphate was mixed with [1-^{12}C]glucose 6-phosphate to give material with close to the natural abundance of ^{13}C at C-1.[8] Since there is no isotope effect on the rate of hydrolysis caused by ^{13}C at C-1, any discrimination between the two species of glucose 6-phosphate results from the ^{18}O-substitution. This discrimination is easily measured by separating glucose 6-phosphate and glucose after half-hydrolysis, and degrading them both separately to ribulose 5-phosphate and CO_2 by the action of glucose-6-phosphate and 6-phosphogluconate dehydrogenases (the glucose was phosphorylated to glucose 6-phosphate by hexokinase and MgATP first). The mass ratio in CO_2 was measured with an isotope ratio mass spectrometer.

The observed ^{18}O isotope effect was 1.013 ± 0.002 (see Table I). When this value was corrected for the isotopic purity of the starting materials and the cube root taken,[9] an isotope effect of 1.0046 was obtained for a single ^{18}O-substitution. The equilibrium

Table I. Secondary ^{18}O Isotope Effects on the Hydrolysis of Glucose 6-Phosphate[a]

	isotope effect calculated from	
fractional reactn	residual glucose 6-phosphate[b]	glucose product[c]
0.4898	1.0162	1.0104
	1.0124	1.0120
0.4721	1.0158	1.0122
	1.0131	1.0119
0.480	1.0113	1.0158
	1.0123	

[a] The isotope effects are for substitution of three ^{18}O in the nonbridge positions of the phosphate group but have not been corrected for the lack of isotopic purity of the starting materials. See ref 9 for the corrections needed here. [b] Calculated from the expression log $(1 - f)$/ log $[(1 - f)(R_s/R_0)]$, where f is fraction of reaction and R_s and R_0 are ^{13}C content of the CO_2 from C-1 in residual glucose 6-phosphate and initial glucose 6-phosphate, respectively. [c] Calculated from the expression log $(1 - f)$/ log $(1 - fR_p/R_0)$, where R_p is the ^{13}C content of C-1 from product glucose.

(1) Benkovic, S. J.; Schray, K. J. *Enzymes (3rd Ed.)* **1973**, *8*, 201. Westheimer, F. H. *Chem. Rev.* **1981**, *81*, 313. Westheimer, F. H. *Acc. Chem. Res.* **1968**, *1*, 70.

(2) Bourne, N.; Williams, A. *J. Am. Chem. Soc.* **1984**, *106*, 7591.

(3) Skoog, M. T.; Jencks, W. P. *J. Am. Chem. Soc.* **1984**, *106*, 7597.

(4) This analysis focuses on bond stretches and ignores the loss or decrease in bending frequencies that results from an exploded transition state with low axial P–O bond order. The quantitive calculations we have made take bending modes into account.

(5) Halman, M.; Degani, Ch. *J. Am. Chem. Soc.* **1966**, *88*, 4075.

(6) Bunton, C. A.; Chaimovich, H. J. *J. Am. Chem. Soc.* **1966**, *88*, 4082.

(7) O'Leary, M. H.; Marlier, J. F. *J. Am. Chem. Soc.* **1979**, *101*, 3300.

(8) Having the mass ratio close to the 1.1% natural abundance value minimizes errors from contaminating CO_2 during the analysis.

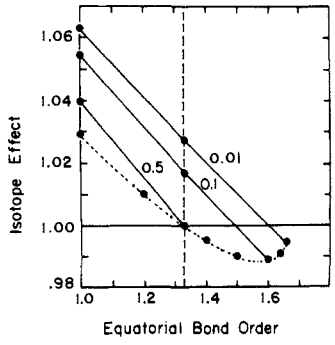


Figure 1. Calculated kinetic ^{18}O isotope effects at 100 °C for phosphate transfer. The reactant is a phosphate monoester with three ^{18}O in the nonbridge oxygens. The transition state is a trigonal bipyramid with the equatorial bond order shown. The vertical dashed line is the equatorial bond order in the reactant before reaction, and the dotted line connects points where the total bond order to P is 5 in the transition state. Solid lines connect points with the same axial bond order (numbers shown on figure).

^{18}O isotope effect for deprotonation of a phosphate monoester was determined in the previous paper[11] to be 1.0042 per ^{18}O at 100 °C, and thus correction for the preequilibrium proton transfer to the bridge oxygen in mechanisms 1 or 3 gives 1.0004 as the isotope effect on P–O bond cleavage (1.001 for three ^{18}O).

We show in Figure 1 isotope effects for phosphate ester hydrolysis calculated at 100 °C for ^{18}O-substitution in the three nonbridge oxygens.[12] If the mechanism is dissociative as believed, the observed isotope effect near unity suggests that total bond order to P is *not* conserved in the transition state. For an axial bond order of 0.1, the equatorial bond order would be 1.48, which corresponds to a total bond order to P of 4.64, or a net positive charge on P of 0.36. For an axial bond order of 0.01, the equatorial bond order would be 1.59, and the charge on P +0.21. The alternate explanation that the transition state is early is not possible in this case because the equilibrium constant for the reaction is near unity if the concentration of water is expressed in the same units as for the other reactants. These data do not address the question of whether metaphosphate is a free intermediate or whether the reaction is an S_N2 one with very low axial bond order in the transition state. Because K_{eq} is near unity and metaphosphate is very unstable relative to reactant or product, the transition states would be very similar for both mechanisms.

Acknowledgment. This work was supported by NIH Grant GM 18938 to W.W.C. W.B.K. holds a NIH postdoctoral fellowship (GM09677). NSF provided a major portion of the funding to purchase the isotope ratio mass spectrometer (PCM 8218027). We thank Isabel Treichel for technical assistance with isotope ratio mass spectrometry.

(9) The equation used to calculate ^{18}k (the ^{18}O isotope effect for single ^{18}O-substitution) was [10]

$$^{18}k = 1 + ([w/(^{13}k - [(1 - b)z/(bx)][w - {}^{13}k])]^{1/3} - 1)[1 + (1 - y)/3]$$

where w = observed isotope effect = 1.013, x = fraction of ^{13}C in the [1-^{13}C]glucose = 0.99, y = fraction of $^{18}O_3$ in the ^{13}C,^{18}O-containing glucose 6-phosphate = 0.85, z = fraction of ^{13}C in [1-^{12}C]glucose = 0.0001, b = fraction of ^{13}C,^{18}O-containing glucose 6-phosphate in the final mixture (natural abundance is ~0.0111) = 0.011 75, and ^{13}k = ^{13}C isotope effect at C-1 from analogous hydrolysis experiments with natural abundance glucose 6-phosphate where both residual glucose 6-phosphate and glucose product were analyzed. The measured value was 1.002 ± 0.002; we have assumed this to be unity.

(10) Hermes, J. D.; Morrical, S. W.; O'Leary, M. H.; Cleland, W. W. *Biochemistry* **1984**, *23*, 5479.

(11) Knight, W. B.; Weiss, P. M.; Cleland, W. W., preceding article in this issue.

(12) Calculations were carried out with the BEBOVIB-IV program of Sims.[13] The reactant model was a phosphate group with a carbon attached, and the transition-state model was a symmetrical trigonal bipyramid, since K_{eq} is near unity for glucose 6-phosphate hydroiysis, if the concentration of water is expressed in the same units as the other reactants. Force constants for single bonds were assumed to be 4.5 mdyn/Å for P–O stretching and 1.9 mdyn Å/radian^2 for O–P–O bending, with 16% off-diagonal coupling between P–O stretches and 23% off-diagonal coupling between bends sharing a common bond, since these values correctly reproduce the Raman frequencies for PO_4^{3-} (Kohlrausch, K. W. F. *Ramanspectren*, reprinted by Edwards: Ann Arbor, MI, 1945. Hanwick, T. J.; Hoffman, P. *J. Chem. Phys.* **1949**, *17*, 1166). The program adjusts these force constants for changes in bond order and bond angles. In the transition-state model, the axial P–O stretches were coupled in off-diagonal position by a factor of 1.1 so that the asymmetric stretching vibration became the reaction coordinate motion with a negative frequency. These calculations should be reliable for axial bond orders of 0.01 or above, but lower axial bond orders reduce the frequency of the symmetrical out of plane wag below a value that is reasonable for the out of plane wag in metaphosphate (i.e., below 200 cm^{-1}). The frequencies of the other three vibrational modes of a trigonal PO_3 unit approach asymptotic limits as the axial bond order is reduced to zero in our transition-state model.

(13) Sims, L. B.; Burton, G.; Lewis, D. E. BEBOVIB-IV, Program No. 337, Quantum Chemistry Exchange Program, Department of Chemistry, Indiana University, Bloomington, IN 47401.

Stereochemistry of the Terminating Methyl → Methylene Elimination in Kaurene Biosynthesis

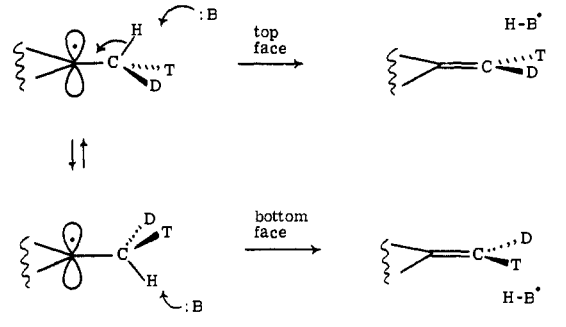
Robert M. Coates,* Stacie Canan Koch, and Shridhar Hegde

Department of Chemistry, University of Illinois Urbana, Illinois 61801

Received November 25, 1985

The exocyclic methylene groups commonly found in naturally occurring terpenes presumably arise by regiospecific methyl → methylene eliminations that terminate enzyme-catalyzed cyclizations. There is also a stereochemical option associated with these eliminations specified by the position of the basic group within the active site of the cyclase. The stereospecificity of the methyl → methylene elimination may be elucidated by use of chiral methyl groups.[1] We disclose herein results[2] that establish the stereospecificity of the terminating elimination in the cyclization of geranylgeranyl pyrophosphate (**2**) to kaurene (**4**) catalyzed by an enzyme extract from *Marah macrocarpus* seeds.[3]

Stereochemical Options for the Methyl/Methylene Transformation



Reduction of (*R*)- and (*S*)-β-deuteriostyrene oxide[4] with lithium triethylborotritide[5] (THF, 25 °C, 2 h) afforded (1*S*,2*R*)- and (1*R*,2*S*)-[2-^{2}H,^{3}H]-1-phenylethanol (61 and 70 mCi; 58 and 64 mCi/mmol). Successive oxidation of diluted portions with chromic acid and trifluoroperoxyacetic acid gave phenyl acetate (14–15 mCi, 71–76%) which was saponified to (*R*)- and (*S*)-sodium

(1) (a) Floss, H. G.; Tsai, M.-D.; Woodward, R. W. *Top. Stereochem.* **1984**, *15*, 253–321. (b) Floss, H. G.; Tsai, M.-D. *Adv. Enzymol.* **1979**, *50*, 243–302.

(2) Presented in part of the Second International Symposium on Synthesis and Applications of Isotopically Labelled Compounds, Kansas City, MO, Sept 5, 1985.

(3) (a) Upper, C. D.; West, C. A. *J. Biol. Chem.* **1967**, *242*, 3285–3292. (b) Oster, M. O.; West, C. A. *Arch. Biochem. Biophys.* **1968**, *127*, 112–123. (c) Schechter, I.; West, C. A. *J. Biol. Chem.* **1969**, *244*, 3200–3209.

(4) (a) Preparation of (*S*)-β-deuteriostyrene oxide, $[\alpha]^{23}$ −43° (*c* 0.9, C_6H_6), optical purity ≥95% by ^{1}H NMR, from the racemate: (a) (*S*)-(1-phenylethyl)amine, 100 °C; (b) recrystallization; (c) CH_3I, $KHCO_3$, ethanol, 25 °C; (d) KOtBu, THF, reflux (30–32% overall). (b) All compounds (except high specific activity intermediates) were characterized by appropriate IR and ^{1}H NMR spectra. Satisfactory elemental analyses were obtained for new compounds by combustion or high-resolution MS.

(5) Coates, R. M.; Hegde, S.; Pearce, C. J. *J. Chem. Soc., Chem. Commun.* **1983**, 1484–1485.