substitution products are obtained.[12] Clearly, this special high pressure technique is the method of choice for the synthesis of these heterobicyclic materials.

In contrast to the success achieved with the acrylic dienophiles, the crotonic dienophiles gave relatively low yields of cycloadducts (entries 5-8). The reductions in yields are attributable to the fact that the β-methyl group introduces steric hindrance to the transition state and also inductively contributes electron density to the dienophile, thus acting in opposition to the electron-withdrawing α-substituent and retarding the rate of reaction. When two β-methyl groups are incorporated on the dienophile (as with methyl senecioate or mesityl oxide) no reactions were observed. The argument employed for the results with the crotonic dienophiles also serves to rationalize the low yields of cycloadducts obtained with the methacrylic dienophiles (entries 9 and 10). Additional evidence in support of the electronic contribution of a β-substituent is available from entries 11 and 12. In these reactions the β-carboalkoxy group enhances the dienophilicity by induction, and appropriately, excellent yields of cycloadducts were obtained;[13] for the thermal reactions the conversions are quite inefficient.[14] In addition, under the high pressure conditions employed the stereochemical integrity of the dienophile was maintained in the cycloadducts: only the endo-cis isomer was produced from dimethyl maleate and furan, and the trans isomer was the sole product from diethyl fumarate and furan. Unlike the high yields obtained with dimethyl maleate or diethyl fumarate and furan, no reactions were detected with furan and either *cis*- or *trans*-1,2,dichloro- or tetrachloroethylene.[15]

Finally, the effects of substituents on the furan nucleus were studied. For the reactions involving 2,5-dimethylfuran (entries 13-16) good yields of adducts (as approximately 1:1 ratios of exo/endo isomers) were realized. In these cases the methyl groups inductively augment the reactivity of the diene by rendering it more electron rich than furan itself, but they also impede the cycloaddition process in a steric fashion; the net result is that the yields of adducts from furan or 2,5-dimethylfuran with acrylic dienophiles are similar, implicating that the electronic and steric effects of the methyl groups are of comparable magnitude.[16] We have also investigated the reactions of furfural and methyl furoate with various dienophiles; both electron rich (i.e., ethyl vinyl ether) and electron poor (i.e., dimethyl acetylenedicarboxylate) dienophiles failed to engage in cycloadditions with these dienes, only starting materials being recovered.

In conclusion, the utilization of pressures in the 8-20 kbar range has proved to be an extremely valuable technique for effecting chemical reactions in which the products are thermally labile (such as **1**).

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(1) This research was supported by National Science Foundation Grant No. MPS 73-04662. The authors thank Professor G. Jura for the hydraulic press equipment used in the present investigation.

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Received December 22, 1975

Solvent Isotope Effect in Inorganic Pyrophosphatase-Catalyzed Hydrolysis of Inorganic Pyrophosphate

Sir:

Phosphate monoester dianions are generally thought to hydrolyze via an SN1 (P), metaphosphate, mechanism characterized by, among other properties, a kinetic solvent isotope effect of, or close to, one.[2] We report here kinetic solvent isotope studies on the simple, Mg^{2+}-catalyzed, and yeast inorganic pyrophosphatase (PPase) (EC 3.6.1.1)-catalyzed hydrolysis of inorganic pyrophosphate (PP_i), and on some detailed models for the mechanism of the enzyme-catalyzed reaction suggested by our results.

The pH-rate profile for PPase at saturating levels of PP_i and high Mg^{2+} concentration shows a bell-shaped curve (Figure 1), with a maximum at pH 7.05 ± 0.05, in accord with previous reports.[3] The pD-rate profile is similar, but the maximum is shifted to pD 7.50 ± 0.05. Shifts of this magnitude are common in comparing pH- and pD-rate profiles.[5a] In the ranges studied, comparison of k_{obsd} at pH equal to X with k_{obsd} at pD equal to $X + 0.50$ yields an average solvent isotope effect of 1.90 ± 0.05. Simple PPi hydrolysis (Figure 2) shows no isotope effect. The shape of the pH-rate profile is in accord with previous results which have been interpreted as showing that PPi trianion is less reactive than dianion, while tetranion is essentially inert.[6] The pH-rate profile for PPi hydrolysis in the presence of Mg^{2+} (Figure 2) is more complex and is discussed in detail elsewhere.[7] For the present discussion the important region is pH 6.0-7.0. Using known thermodynamic parameters for both H^+ and Mg^{2+} dissociation from PP_i,[8] it is possible to show that the rise in k_{obsd} between 6.0 and 6.5 coincides with formation of $MgP_2O_7^{2-}$ and $Mg_2P_2O_7$ so that the plateau region (6.5-7.0) in H_2O should correspond to hydrolysis of either or both of the species. Comparing k_{obsd} in the H_2O plateau region with k_{obsd} in the D_2O plateau region (pD 7.0-7.7) gives a solvent isotope for hydrolysis of the Mg^{2+} complex(es) of PPi of 1.45 ± 0.05.[9] To our knowledge this is the first significant kinetic solvent isotope effect

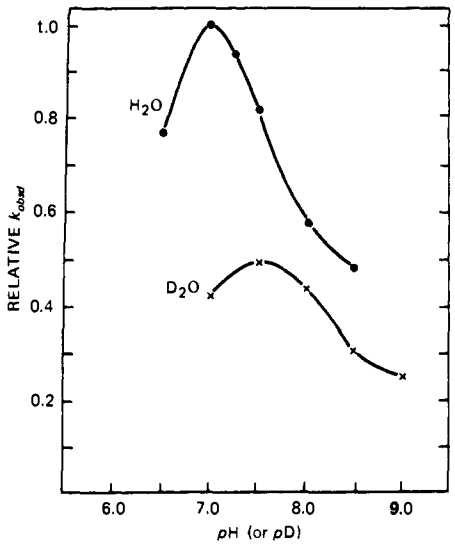

Figure 1. pH- and pD-rate profiles for PPase catalysis of PP_i hydrolysis. Reaction mixture: 1.0 mM Na_4PP_i, 5.0 mM $MgCl_2$, 0.1 M Tris-HCl, 0.20 M KCL. Temperature was 30.0 ± 0.2°. PPase (specific activity 35-40 Kunitz[3a] units/mg) was prepared, and hydrolysis rates (phosphate analysis) were determined as previously reported.[4] pH variation during the course of reaction did not exceed 0.08 pH units and was usually below 0.03 pH units. Constants reported are the average of four to six determinations.

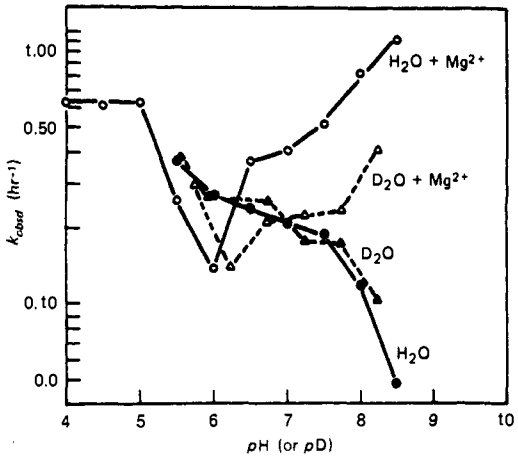

Figure 2. pH- and pD-rate profile for PP_i hydrolysis in the presence and absence of Mg^{2+}. Temperature was 95.0 ± 0.2°. Buffers used were KOH, acetic acid (pH 4.0-5.5), Tris-HCl (pH 6.0-8.0), and KOH, H_3BO_3 (pH 8.5). Concentrations in reaction mixture were 0.10 M buffer, 0.20 M KCl, and 1 mM PP_i for kinetics without Mg^{2+}. For kinetics with Mg^{2+} concentrations used were 0.02 M buffer, 0.28 M KCl with 1 mM Mg(II), and 0.05 mM PP_i at pH 4.0-6.0. At higher pH, 0.06 mM Mg^{2+} and 0.03 mM PP_i were used in order to avoid precipitation problems. Below pH 6, rate constants were determined from initial rates (<10% of reaction). Rate constants are the average of two or three determinations. At 95°, pD = pH_{read} + 0.126.[5b]

reported for hydrolysis of a phosphate monoester dianion, although an effect of this magnitude has been found for a diester monoanion.[10]

Further information on the solvent isotope effect for the enzymatic reaction was obtained via application of the proton inventory technique. This technique consists of measuring the reaction rate in mixtures of H_2O and D_2O where the observed rate constant at an atom fraction of deuterium n, k_n, is given by eq 1,

$$k_n = k_0 \frac{\pi_i(1 - n + n\phi_i^T)}{\pi_j(1 - n + n\phi_j^R)} \quad (1)$$

where k_0 is the observed rate constant in H_2O and ϕ_i^T and ϕ_j^R are the isotopic fractionation factors of the ith exchangeable hydrogenic site in the transition state and the jth exchangeable hydrogenic site in the reactant state, respectively.[11] The proton inventory for PPase was measured between the two maxima of the H_2O and D_2O pH (D)-rate profiles, from pH 7.00 to pD 7.50 (Figure 3). Since pD = pH_{read} + 0.4,[5a] pH_{read} was increased by 0.01 pH unit (base addition) for each incremental rise of 10% in D_2O content. Two simple parameter sets were found to fit the data within experimental error: in set I, $\phi_1^T = 0.40$, $\phi_1^R = \phi_2^R = 0.90$, and all other ϕ values equal 1.00; in set II, $\phi_1^T = 0.37$, $\phi_2^T = 1.37$, and all other ϕ values equal 1.00. Below we consider (Figure 4) three plausible models for the transition state in PPase-catalyzed PP_i hydrolysis which are consistent with what is known about phosphoryl transfer in general and the mechanism of PPase in particular and are in accord with either set I or set II.

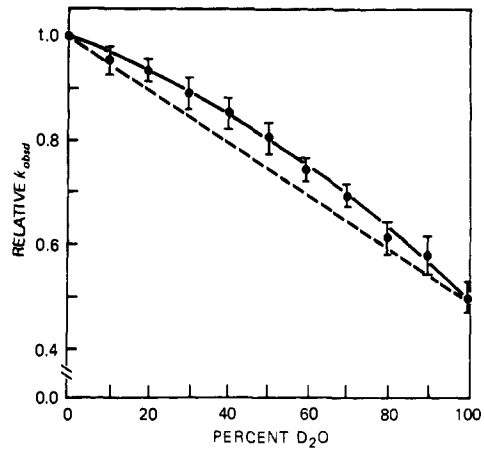

Figure 3. k_n for PPase in mixtures of H_2O and D_2O. Details as in legend for Figure 1. H_2O content of solutions was determined by NMR and density measurements.[12] Rate constants are the average of seven-ten determinations, with average deviations indicated. Solid line is calculated using parametric set I. A line calculated from set II fits the points about as well.

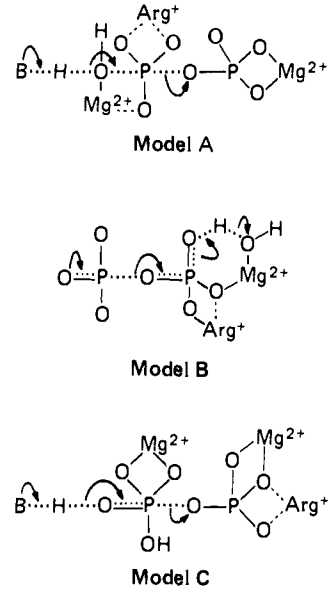

Figure 4. Plausible models for the transition state in PPase catalysis of PP_i hydrolysis.

Model A depicts an SN2 (P) reaction in which Mg^{2+} and a basic group on the protein (possible carboxylate[13]) activate a water molecule toward attack on pyrophosphate. Attack by the incipient anionic oxygen on the phosphoryl dianion is aided by the charge-shielding[14-16] resulting from formation of an arginine-phosphate salt bridge[13a,17] and

the leaving phosphoryl group is activated via Mg^{2+} complexation with a resultant pK_a lowering.[15] Detailed kinetic analysis has shown Mg_2PPi to be the true PPase substrate in a stoichiometric, though not necessarily a structural sense.[3a,18] The two ϕ^R values of 0.9 are well accounted for by a water molecule coordinated to Mg^{2+}, whose protons should each have a ϕ value lying between 0.69, the value for the hydrated proton,[19] and 1.00. In the transition state, a ϕ value of 0.4 for the proton in transit is similar to what has been found in comparable situations,[11b,20] whereas the proton remaining attached to the oxygen resembles more closely a proton in a neutral water molecule and can be assigned a value of 1.00. Model B depicts a pure SN1(P) reaction in which extreme leaving group activation is achieved through coordination to arginine and to Mg^{2+}, and to general acid catalysis via a water molecule bound to Mg^{2+}. The proposed transition state could accommodate a second Mg^{2+} if the two Mg^{2+} functions were divided between two different Mg^{2+} ions. The expected ϕ values are again in accord with set I, with two ϕ^R values of 0.9 for water bound to Mg^{2+}, ϕ^T values of 0.4 for the proton in transit, and 1.00 for the proton remaining with the water oxygen. Model C depicts an addition-elimination reaction in which the transition state occurs during a base-catalyzed breakdown of the trigonal bipyramid formed via water addition and rapid internal proton transfer. The trigonal bipyramid is stabilized via bidentate coordination to Mg^{2+},[16,21,22] and coordination to arginine (and possibly a second Mg^{2+}) activates the leaving group. The expected ϕ values may be in accord with set II. All ϕ^R values should be 1.00, and a ϕ^T value of 0.37 for the proton in transit is reasonable. The uncertainty comes in assigning a ϕ^T value of 1.37 to the proton bound to the pentoxyphosphorane, which must be considered speculative since it is not based on any direct precedent. Such a value would not, however, be unreasonable, given the obvious structural analogy between gem diols (ϕ = 1.23-1.28)[23] and pentoxyphosphoranes and the expected strongly basic nature of the position to which the proton is bound.[24]

Given the uncertainties inherent in solvent isotope studies on enzymatic mechanisms,[25,26] it is clear that the experiments presented here do not allow any final conclusions to be reached. Their value lies rather in defining the simplest allowed mechanisms whose validity can then be tested by other approaches. Within this limited context, our results provide evidence for involvement of at least one water molecule in the transition state. They also hint at a direct catalytic role for Mg^{2+}. Thus, both PPase and Mg^{2+}-catalyzed PPi hydrolysis show appreciable solvent isotope effects, whereas simple PPi hydrolysis does not, and at least, for parameter set I, the ϕ values obtained are fully consistent with water bound to Mg^{2+} as an important part of the transition state.

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Received December 15, 1975

Nature of the Detectable Intermediate in the Permanganate Oxidation of *trans*-Cinnamic Acid

Sir:

In recent papers,[1,2] Lee and Brownridge have reported on the stopped-flow detection of an intermediate (at 415 nm) in the oxidation of *trans*-cinnamic acid (TCA) by acidic permanganate. The conclusion has been made that this intermediate is a relatively stable hypomanganate ester. We wish to report the results of experiments performed by the combined stopped-flow-chemical quenching technique, which show that the oxidation state of manganese in the above intermediate cannot be +5. The scheme of the setup is shown in Figure 1. Syringes S_1-S_3 are discharged at constant speed. In the two-jet mixing chamber, M_1, aqueous TCA is mixed with acidic permanganate. The reacting solution passes down the capillary tube T to a four-jet mixer (M_2), where it is mixed with the quenching solution (aqueous NaI, with starch added in some experiments). Mixer M_2 is part of a stopped-flow instrument with an observation cell at C. Discharge of syringes S_1-S_3 through the two-stage mixing system permits one to (i) monitor the reaction of the short-lived intermediate accumulating in tube T with the quenching reagent and analyze the effluent chemically or (ii) follow the decay of the intermediate without the quenching reagent (aqueous $HClO_4$ in S_3).

Prior to the runs on the two-stage system, stopped-flow experiments were performed to determine the time at which the 415-nm trace reaches its minimum (% *T*). By varying the flow rate, the length and diameter of capillary T, the residence times in T were set as close as possible to these values. Thus the concentration of the intermediate at M_2 was a maximum in each run. The reaction between starch and NaI/I_2 was found to be complete within the time of mixing; therefore, starch could be used to enhance the sensitivity of detection of iodine at 415 nm.

The traces in Figure 1 illustrate the efficiency of quenching. The system is originally filled with water, which is discharged up to point 1. In section 1-2, the reacting solution arrives, while in section 2-3 it flows through the observation cell. At point 3 the flow stops. Trace I shows the decay of