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Phosphate Monoester Hydrolysis in Cyclohexane

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With an estimated half-life of 10^{11} years at 25 °C, the spontaneous hydrolysis of a monoalkyl phosphate dianion may be the most difficult reaction that is catalyzed by an enzyme.[1] At the active sites of hydrolytic enzymes, this reaction proceeds with a half-life of 17 ms.[1] It has been suggested,[2] although not universally agreed upon,[3] that the extraction of substrates from solvent water may be among the factors that contribute to the rate enhancements produced by enzymes in general. Abell and Kirby's demonstration[4] that the hydrolysis of 4-nitrophenyl phosphate proceeds $>10^6$-fold more rapidly in wet DMSO than in water and subsequent observations by Hengge and his associates[5] furnish experimental support for the possibility that desolvation effects figure prominently in the action of phosphate monoesterases. Here, we show that the dianion of neopentyl phosphate (NP^{2-}) enters wet cyclohexane as its tetrabutylammonium (TBA^+) salt. In cyclohexane, the second-order rate constant for phosphomonoester hydrolysis is enhanced by a factor of 2.5×10^{12} compared with the second-order rate constant for hydrolysis of NP^{2-} in water.[7]

Distribution coefficients were determined by stirring a small volume (1 mL) of aqueous NP^{2-} (0.1 M, titrated to pH 11 with TBA hydroxide) for 6 h with a large volume (100 mL) of cyclohexane and then back-extracting the organic layer into a small volume (0.65 mL) of D_2O (Supporting Information (SI)) for analysis by proton NMR. The distribution coefficient observed for NP^{2-} transfer from water to cyclohexane in the presence of excess TBA^+ was $7.0\ (\pm 0.4) \times 10^{-6}$ at 25 °C, and TBA^+ was found to be present in cyclohexane in a molar ratio of 2 parts TBA to 1 part of NP. This value was unaffected by varying the concentrations of solutes in the aqueous phase, implying that TBA salts are fully dissociated in cyclohexane.[6] Thermodynamic changes associated with water-to-cyclohexane transfer were determined from a van't Hoff plot of these distribution coefficients as a function of changing temperature in the range from 10 to 50 °C, which was linear over the range examined. The distribution coefficient increased with increasing temperature (SI).

To determine the rate of monoester hydrolysis in wet cyclohexane, portions (10 mL) of the clear cyclohexane layer, prepared at 25 °C as described above, were incubated in Teflon-lined acid digestion bombs for various time periods. After heating, samples in cyclohexane (10 mL) were back-extracted into water (1 mL), the aqueous phase was evaporated to dryness (any neopentanol was removed by that process), and the residue was dissolved in D_2O containing dioxan as an integration standard, for analysis by NMR. The course of hydrolysis was monitored by comparing the integrated intensity of the peak arising from NP^{2-} in the starting material and reacted samples. Hydrolysis was also monitored by the release of inorganic phosphate, measured spectrophotometrically using acid-molybdate,[7] with identical results. Hydrolysis was found to proceed with first-order kinetics under all conditions examined. Rate constants obtained for the hydrolysis of NP^{2-} over the temperature range between 74 and 112 °C yielded linear Arrhenius plots (Figure 1) from which rate constants at 25 °C and the corresponding thermodynamics of activation were estimated (Table 1).

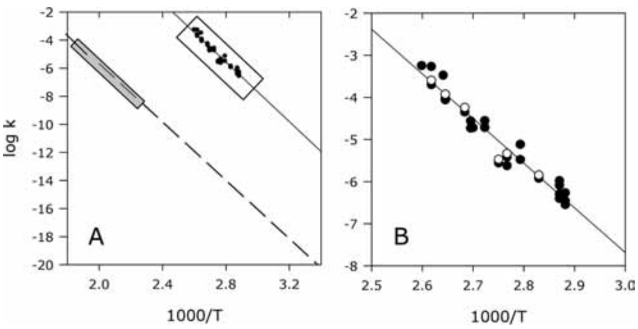

Figure 1. Arrhenius plots showing the hydrolysis of NP^{2-} in cyclohexane. Observed first-order rate constants (k) are not corrected for water concentration. For comparison, the rates of reaction in water are shown as a broken line with the temperature range over which data were collected indicated by the gray box (A). Data obtained by NMR are shown as solid points, and data collected by the molybdate assay for inorganic phosphate are shown as open points (B). In A, the vertical axis on the right corresponds to 25 °C.

In wet cyclohexane, NP^{2-} hydrolysis proceeded with a rate constant of $3.8 \times 10^{-12}\ s^{-1}$ at 25 °C, exceeding the estimated rate constant for hydrolysis of the dianion in water $(2 \times 10^{-20}\ s^{-1})^1$ by a factor of 1.9×10^8. Although it is possible that NP^{2-} retains waters of hydration in cyclohexane,[8] experiments involving a mixture of NP^{2-} in wet cyclohexane with varying amounts of dry cyclohexane showed that the rate of hydrolysis of NP^{2-} in cyclohexane varied in proportion to the concentration of water that was present (Figure 2). This implies that hydrolysis is bimolecular, as it is for the aqueous reaction (in water, phosphate monoester hydrolysis proceeds through a concerted, dissociative transition state).[9] With the concentrations of water present in water-saturated cyclohexane $(4.2 \times 10^{-3}\ M)^{10}$ and pure water (55.5 M) corrected for, the second-order rate constant for water attack on NP^{2-} in cyclohexane ($8.8 \times 10^{-10}\ s^{-1}\ M^{-1}$) exceeds the rate constant for attack in water ($3.6 \times 10^{-22}\ s^{-1}\ M^{-1}$) by a factor of 2.5×10^{12}. Remarkably, the source of this rate enhancement is entirely entropic in origin (Table 1).

In water, the monoanions of alkyl phosphate monoesters are hydrolyzed $\sim 10^{10}$-fold more rapidly than their dianions.[1] That special reactivity has been attributed to intramolecular hydroxyl group catalysis of alkoxide elimination through the agency of intervening water molecules.[11] It was therefore of interest to compare the rates of hydrolysis of NP in its uncharged, monoanionic, and dianionic forms, in wet cyclohexane. An aqueous solution of the di-TBA salt of NP^{2-} was adjusted with HCl to various pH values corresponding to different states of ionization of NP (pK_a values 1.8 and 6.8) and extracted with cyclohexane. Proton NMR showed that the number of equivalents of TBA^+ in cyclohexane corresponded to the number of negative charges on the major

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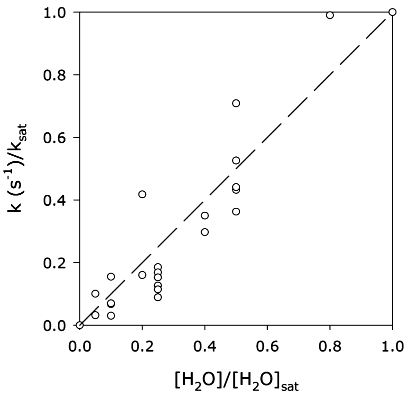


Figure 2. Effect of water concentration on rate of NP^{2-} hydrolysis in cyclohexane at 90 or 100 °C. Water concentration was adjusted by mixing wet cyclohexane (4.3×10^{-3} M H_2O) with dry cyclohexane. Values are normalized relative to the rate of hydrolysis in water-saturated cyclohexane at the appropriate temperature.

Table 1. Rate Constants and Thermodynamics of Activation for Hydrolysis of NP^{2-} in Water[1] and Cyclohexane (Thermodynamic Parameters in kcal/mol)

	$\Delta G^‡$	$\Delta H^‡$	$T\Delta S^‡$	k_{25} (s^{-1})	k_{25} (s^{-1} M^{-1})
H_2O	44.3	47.0	2.7	2.0×10^{-20}	3.6×10^{-22}
CHX	32.9 ± 0.53	47.6	14.7	3.8×10^{-12}	8.8×10^{-10}

ionized form of NP that had been present in the aqueous phase (Table 2). Distribution coefficients observed for NPH_2, NPH^-/TBA^+, and NP^{2-}/$2TBA^+$ were found to be similar, and their rate constants for hydrolysis in wet cyclohexane were distributed over a surprisingly narrow range (Table 2).

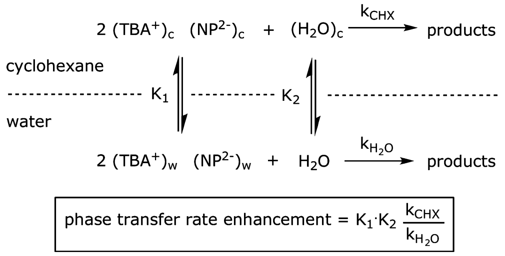
Table 2. Rate Constants for Hydrolysis and Coefficients for Transfer of NP^{2-}, NPH^-, and NPH_2 from Water to Cyclohexane

pH (aq.)	TBA_c/NP_c	species (CHX)	K_1	k_{90} (s^{-1})
0	0	NPH_2	9.9×10^{-5}	3.6×10^{-6}
1.8	0.09	$NPH_2 + NPH^-$	5.0×10^{-5}	6.8×10^{-6}
5.5	1.12	NPH^-	4.7×10^{-6}	1.1×10^{-5}
6.8	1.49	$NPH^- + NP^{2-}$	3.6×10^{-6}	1.8×10^{-5}
12.0	2.0	NP^{2-}	7.0×10^{-6}	1.0×10^{-5}

In wet cyclohexane, the concentration of water (4.2×10^{-3} M) is considerably higher than the concentrations of the neopentyl esters that were present in these experiments (~10^{-6} M). Thus, one might question whether the unexpectedly high apparent reactivity of monoester dianions in wet cyclohexane arises from a small population of monoanions that reacts relatively rapidly and that can be replenished by protons donated by H_2O. The similarity of the observed rate constants for hydrolysis of NPH_2, NPH^-, and NP^{2-} in wet cyclohexane seems to render that explanation unlikely. Transfer to cyclohexane may level the observed rate constants by removing the intervening water molecules that have been postulated to participate in intramolecular general acid catalysis,[11] tending to eliminate the special reactivity of monoester monoanions.

For an enzyme acting by desolvation effects, substrate binding might be compared to solvent transfer in the presence of a phase transfer catalyst. Scheme 1 shows that for such a catalyst to be effective, $K_1 \cdot K_2 \cdot k_{CHX}$ must exceed the value of k_{H2O}. In the reactions described here, the resulting "phase transfer rate enhancement," as defined in Scheme 1, is 1.3×10^3-fold for NP^{2-} hydrolysis at 25 °C, and that value increases with increasing temperature (see SI for thermodynamic details).

Scheme 1. Phase Transfer and NP Hydrolysis in the Cyclohexane/Water System



The present findings imply that the distribution coefficient of phosphate esters from water to wet cyclohexane increases greatly as they proceed from the ground state to the transition state in their hydrolysis. As has been suggested for nucleophilic displacement reactions in the vapor phase,[12] it seems likely that the present solvent effects arise in part from greater delocalization of charge in the transition state than in the ground state. Possible changes in transition state structure, and the extent to which water molecules remain associated with phosphate esters when they are transferred from water to cyclohexane, remain to be determined.

These rate enhancements approach or surpass the rate enhancements produced by many hydrolytic enzymes and accord with the possibility that desolvation plays a substantial role in the action of phosphate monoesterases.

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Supporting Information Available: Detailed kinetic methods; van't Hoff analysis of equilibria for transfer of NP^{2-} and NPH^- from water to cyclohexane; effect of ester concentration on distribution coefficients observed for transfer of NP^{2-}, NPH^-, and NPH_2 from water to cyclohexane; error analysis for distribution coefficients and rate constants; estimation of $\Delta H^‡$ values for phase transfer catalysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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