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An Assessment of Desolvation on Rates of Acetyl Transfer: Insights into Enzyme Catalysis

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Abstract: Enzymes greatly enhance the rate of reactions by a variety of physical organic mechanisms. One of the more contentious of these has been desolvation. To get a quantitative assessment of this contribution, we examined acetyl transfer to oxydianions. This is a model reaction for enzymes in which a high-energy acyl phosphate is formed. The aqueous reaction of phosphate or phosphonates with *p*-nitrophenyl acetate (pNPA) shows a substantial negative deviation from the Brønsted correlation obtained with monoanionic nucleophiles and from the reactivity of other, larger, oxydianions (molybdate, arsenate and vanadate). This, and other data, suggests a significant contribution of desolvation to the activation energy. To further investigate this we studied the effect of various DMSO (dimethyl sulfoxide)/H_2O mixtures on the reaction of chloromethylphosphonate, and of molybdate, on the reaction with pNPA. Increasing the DMSO concentration from 1% to 90% (v/v) increases the second-order rate constant for each of these reactions by over a factor of 5000. This is over a 1000 times greater than the enhancing effect on the reaction of phenoxides and over 10^5 times the (inhibiting) effect on the reaction with neutral nucleophiles (imidazole). Extrapolation to pure DMSO yields a rate enhancement of $\sim 10^5$, relative to the reaction in water, for the oxydianions. This enhancement is over 3 orders of magnitude greater than that seen with monoanionic phenoxide nucleophiles. This suggests a significant role for desolvation in the reactions in the enzyme-catalyzed nucleophilic reactions of inorganic phosphate but a modest role in reactions with less highly charged nucleophiles.

Enzymes are responsible for enormous rate enhancements. Depending on how this catalytic power is calculated,[1,2] rate accelerations of 10^8−10^{15} are typical [k_{cat}/k_{non} values, where k_{non} is the rate constant for the, often unobserved, nonenzymatic reaction in aqueous solution corresponding mechanistically to the enzyme-catalyzed reaction; the current record for this ratio is 10^{17} for orotidine 5′-phosphate decarboxylase, or a value of 10^{23} M^{-1} for $(k_{cat}/K_m)/k_{non}$, the "catalytic proficiency" of the enzyme[2]]. There is no question that enzymes achieve this impressive catalytic efficiency through a variety of physical organic mechanisms (e.g., nucleophilic and/or general acid−base catalysis, substrate binding in proper proximity and orientation, electrostatic catalysis, solvation, and desolvation). However, just how much each of these contribute (and indeed, how separate they are) has remained a contentious issue.[3,4] This study was undertaken to quantitatively evaluate the contribution of desolvation to enzymic rate enhancements, a topic which has recently received much renewed interest (e.g.,[5,6]). The model reaction we chose was acetyl transfer from *p*-nitrophenol to oxydianions. Displacement reactions involving oxydianions, particularly phosphate, are of great practical importance in biochemistry. There are over 130 enzymatic reactions in which the phosphate dianion acts as a nucleophile. Particularly important among these are acyl transfer reactions in which a high-energy acyl phosphate intermediate is formed. It has long been known that in aqueous solutions the phosphate dianion is 2 to 3 orders of magnitude less reactive than monoanionic or neutral nucleophiles of comparable basicity.[7] Desolvation of a strongly hydrated species, such as the phosphate dianion, is expected to make a significant contribution to rate constant (k_{cat}) for a phosphate nucleophile bound at the active site of an enzyme. Indeed, it has been shown that even for a monoanion such as Cl^- the reactivity (in the S_N2 reaction with CH_3Br) is strongly dependent on the nature of the solvent. As the solvation of the nucleophile decreases (e.g., from a decrease in the solvent polarity or hydrogen bonding ability) the second-order rate constant is enhanced, relative to that in water, reaching a factor of 10^{16} when the gas-phase reaction is compared to the aqueous reaction.[8] To get an assessment of how much the microsolvent of an enzyme's active site can contribute to the enzymatic rate enhancement (when compared to the reaction in water) and to compare this solvent effect on oxydianions with that on monoanionic and neutral nucleophiles, we investigated the reaction of some phosphate analogues with *p*-nitrophenyl acetate in water and in water/DMSO mixtures. This solvent mixture was chosen for a variety of reasons. DMSO is a nonprotic solvent and does not effectively solvate anions. Furthermore, the dielectric constant of the medium is reduced by only 30% as the mole fraction of DMSO is increased from 0 to 0.7.[9] This later property means that the electrostatic effect of solvent

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variation will be minimized so that the main effect on the rate constant should be that of solvent reorganization as the reaction progresses from the ground state to the transition state.

Unusual Characteristics of the Reaction of Phosphate and Phosphonates with *p*-Nitrophenyl Acetate

The low intrinsic reactivity of esters such as pNPA (*p*-nitrophenyl acetate) with the phosphate dianion[7] is also seen in the reaction with dianionic phosphonates.[10] This could reflect, at least in part, the requirement for removal of a strongly held solvent molecule from a nucleophilic site on the dianion. Consistent with this is the observation that, in the Brønsted relationship between the reaction of monoanionic and neutral nucleophiles with pNPA in water, the phosphate dianion shows a negative deviation (by a factor $\sim 10^2$) from this correlation.[11] When the reaction, albeit with a different set of nucleophiles, is carried out in 28.5% ethanol/water the negative deviation of the phosphate dianion from the correlation line is not as significant.[12] In this later study, Bruice and Lapinski found that phenoxide reacts with pNPA about 10^3 times faster than does the phosphate dianion while the data for the reactions in water indicate that this ratio is about 10^4.

The acyl transfers to phosphonates or phosphate have two other notable features which distinguish them from reactions involving acyl transfer to neutral or monoanionic nucleophiles:

1. Low β_{nuc}: In 1960 Jencks and Carriuolo,[11] extending Bruice and Lapinski's work,[12] showed that the reactivity of a wide variety of nucleophiles toward pNPA can be correlated with their pK_a values. Of these 50 nucleophiles, most were either oxymonoanions or neutral amines and the general trend in reactivity corresponds to a $\beta_{\mathrm{nuc}} \approx 0.8$ (25 °C). Using a more homogeneous series of nucleophiles (phenoxides) Williams[13] obtained a $\beta_{\mathrm{nuc}} = 0.75(\pm 0.04)$ for the reaction with pNPA at 25 °C. This can be compared with a value of $\beta_{\mathrm{nuc}} = 0.3$ for the reaction of a series of phosphonates with either pNPA or its thiol ester analogue at 37 °C.[10] The low β_{nuc}value is also consistent with a significant role of desolvation in the reaction. The more basic the phosphonate the more tightly it is expected to bind water.[14] This will partially compensate for the greater nucleophilic reactivity of the more basic phosphonates.

2. Unusually favorable entropy of activation: The reaction of methylphosphonate with pNPA in aqueous solutions shows a value of $\Delta S^{\ddagger} = -10$ eu.[15] This is considerably less negative than the value usually seen for bimolecular reactions in aqueous solution. For example, in the acetyl transfer from *p*-nitrophenol to acetate [$\Delta S^{\ddagger} = -29$ eu[16]] or to imidazole [$\Delta S^{\ddagger} = -30$ eu[17]] the activation entropies are about 3 times more negative than those for the reaction with the dianionic phosphonate. This is also consistent with a significant contribution of removal of highly ordered water molecules from the phosphonate as the reactants progress toward the transition state.

To more thoroughly examine, and quantitate, this solvation effect on the reactivity of phosphonates as acyl acceptors, we investigated the kinetic effect of DMSO/H_2O mixtures on the reaction.

Experimental Section

Materials. *p*-Nitrophenyl acetate was obtained from Sigma-Aldrich Chemical Co. The buffers (HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid),[1] pH 7−8; BICINE (*N*,*N*-bis(2-hydroxyethyl)-glycine), pH 8−9, and CAPS (cyclohexylaminopropanesulfonate), pH 9−10) were obtained from Sigma-Aldrich Chemical Co. as the free acid. Solutions were adjusted to the desired pH with sodium hydroxide. The phosphonates were either prepared or obtained, as described earlier.[10] Sodium molybdate (dihydrate) was obtained from Matheson, Coleman and Bell and stock solutions (0.5 M) were prepared by dissolving Na_2MoO_4 in buffer solutions (0.01 M HEPES, 0.1 M NaCl) and adjusting the pH to 7.5 with HCl. At this pH there is negligible polymerization of molybdate.[18] Stock vanadate solutions were prepared essentially as described by Gresser et al.[19] by dissolving vanadium oxide (Sigma-Aldrich) in buffer solutions (0.01 M CAPS, 0.1 M NaCl) containing a 4-fold excess of NaOH over V_2O_5 and *carefully* adjusting the pH (8.8 to 9.5) with HCl. At the vanadate concentrations used in this study (<5 mM), the predominant species are $H_2VO_4^-$ and HVO_4^{2-}.[19]

Methods. Aqueous pH measurements were made with a Metrohm (Brinkmann) combititrator. pH and pK_a measurements in H_2O/DMSO mixtures were made by using the overlapping indicator method using the phenols and data of Buncel et al.[20] The phosphonate or molybdate solutions in H_2O/DMSO were prepared by first dissolving the disodium salt in a buffered aqueous solution (10 mM HEPES, pH 7.5 with molybdate and 10 mM CAPS, pH 9.5 with chloromethyl-phosphonate). A stock solution with sodium sulfate was prepared in the buffer at the same concentration as the phosphonate or molybdate. This was then mixed with DMSO and used as the blank when measuring the kinetics for the phosphonate and molybdate reactions. This blank solution was also used to maintain a constant ionic strength when the kinetics were followed by varying the nucleophile concentration. The reaction was followed by measuring the absorbance ($\lambda = 400$ nm for *p*-nitrophenoxide) on a Hewlett-Packard model 8452A diode array spectrophotometer. In water, the reactions were initiated by adding 10 μL of the ester (10 mM in DMSO) to 1 mL of a thermally equilibrated (25 °C) buffer solution (ionic strength maintained at 1.6 M with Na_2SO_4) containing a large excess of the nucleophile. In general the increase in the absorbance was typically followed for at least 4 half-lives and the data analyzed by nonlinear regression to a first-order curve.[21] The internal standard deviations of the pseudo-first-order rate constants were consistently less than 1%. For the reactions in H_2O/DMSO mixtures the solubility of the nucleophile decreases as the DMSO concentration increases. At the highest DMSO concentrations the concentrations of the nucleophile (~2 mM), while still high enough to carry out the reactions under pseudo-first-order conditions, was (in the case of the phosphonate) sufficiently low so that the reactions were followed by the initial rate method (i.e., pseudo-zero-order conditions). At DMSO concentrations ≥60% v/v corrections in the second-order rate constants were made for the fraction of the nucleophile present as the dianion. All rate constants were determined at least in triplicate for each reaction.

Results

Brønsted Correlation. To compare the β_{nuc} for the reaction of pNPA with phosphonates with the corresponding values for the reactions with other nucleophiles we have repeated, at 25 °C, the determination made at 37 °C[10] with a series of

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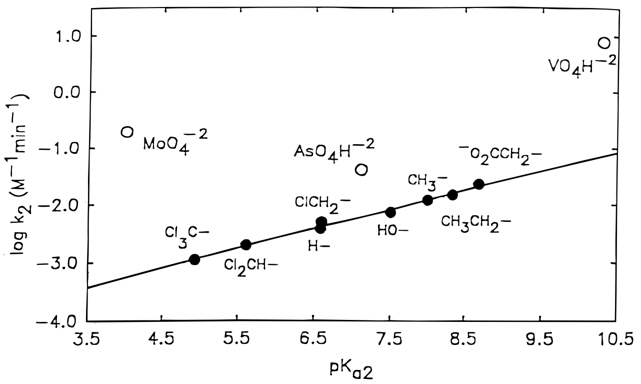

Figure 1. Brønsted correlation of the rate constants for the reaction of a series of phosphonates (R-PO_3^{2-}) with *p*-nitrophenyl acetate in water (1% v/v DMSO, $\mu = 1.6$ M, 25°) yielding a value of $\beta_{nuc} = 0.3$. Also illustrated are the positive deviations from this correlation of some phosphate analogues (○).

phosphonates (Figure 1). We find the following correlation equation:

$$\log k_2\ (M^{-1}\ min^{-1}) = [0.34(\pm 0.02)]pK_{a2} + 5.1(\pm 0.4) \quad [n = 8, r^2 = 0.988] \quad (1)$$

Thus, the β_{nuc} is essentially the same at 25 and 37 °C. Also indicated in Figure 1 are some additional phosphate analogues, all showing **positive** deviations from the correlation line. Particularly notable is molybdate which is 370 times more reactive than a phosphonate of comparable pK_{a2}. Molybdate has been shown to be a nucleophilic catalyst for this reaction.[22]

It will be noted that the phosphate dianion shows no deviation from the correlation line based on the reactivity of the phosphonates in water. This is in contrast to the negative deviation seen in the correlation based on monoanionic oxygen nucleophiles.[11]

Effect of DMSO on Reactivity. The reactions of the dianionic nucleophiles with pNPA could be followed conveniently in DMSO/H_2O mixtures up to about 90% (v/v) (which corresponds to 0.70 mol % DMSO or $[H_2O] = 0.5.6$ M). At DMSO concentrations ≤60% (v/v) the reactions could be followed over a wide enough range of nucleophile concentrations to establish the kinetics to be first-order in acyl acceptor (molybdate or chloromethylphosphonate). The dependence of the second-order rate constant on the water concentration is illustrated in Figure 2. At water concentrations less than 28 M [≈50% (v/v)] the molybdate reaction is more sensitive to the increasing DMSO concentration than is the phosphonate reaction and both of these are more sensitive than are the reactions of various phenoxides with pNPA.[20] Furthermore, once the DMSO concentration exceeds 50% (v/v) ($\log[H_2O] < 1.44$) there appears to be a linear relationship between log k_2 and the decreasing $\log[H_2O]$. This dependence of k_2 on the water concentration ($5.6\ M \leq [H_2O] \leq 27.9$ M which corresponds to a mole fraction of DMSO between 0.7 and 0.2) is given below for each of the oxydianions. The data of Buncel et al.[20] for 4-cyanophenolate are included for comparison:

For 4-cyanophenolate (pK_a in $H_2O = 7.95$)

$$\log k_2(M^{-1}\ min^{-1}) = -[1.91(\pm 0.08)]\log[H_2O] + 2.85(\pm 0.09) \quad [r^2 = 0.997] \quad (2a)$$

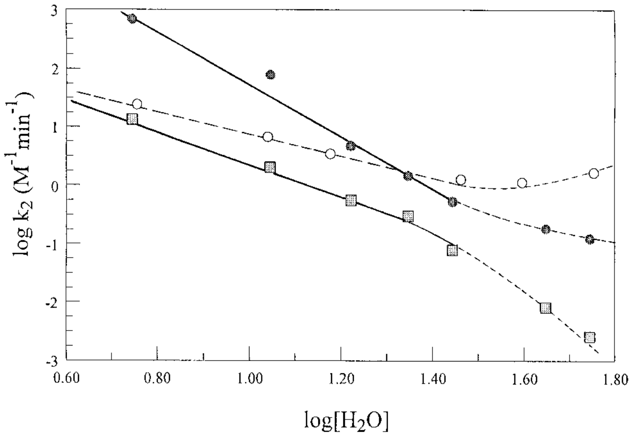

Figure 2. Dependence of the rate constants for the reaction of $ClCH_2PO_3^{2-}$ (■) and of MoO_4^{2-} (●) with *p*-nitrophenyl acetate on the log of the water concentration in H_2O/DMSO solutions (25 °C). Also illustrated are the data of Buncel et al.[20] for the reaction of 4-cyanophenolate with pNPA (○).

For chloromethylphosphonate (pK_{a2} in $H_2O = 6.6$)

$$\log k_2(M^{-1}\ min^{-1}) = -[3.0(\pm 0.2)]\log[H_2O] + 3.5(\pm 0.3) \quad [r^2 = 0.984] \quad (2b)$$

For molybdate (pK_{a2} in $H_2O = 3.8$)

$$\log k_2(M^{-1}\ min^{-1}) = -[4.6(\pm 0.4)]\log[H_2O] + 6.4(\pm 0.4) \quad [r^2 = 0.982] \quad (2c)$$

In contrast to the reaction with these anionic nucleophiles, the reaction of imidazole with pNPA was found to be inhibited by increasing DMSO concentrations. Thus, when the DMSO concentration was increased from 1% to 90% (v/v) the second-order rate constant for the reaction **decreased** 20-fold. This is in contrast to the reaction of pNPA with 4-cyanophenylate, which shows a 15-fold **increase** in the rate constant over this DMSO concentration range,[20] and 5200- and 5500-fold increases with $ClCH_2PO_3^{2-}$ and MoO_4^{2-}, respectively.

Effect of DMSO on Acidity. There is a linear increase in the pK_{a2} of chloromethylphosphonate and of molybdate with increasing mole fraction of DMSO ($0 \leq \chi \leq 0.7$). As has been observed for the series of phenols studied by Buncel et al.,[20] the sensitivity of the pK_a to increasing DMSO concentration is proportional to the pK_a of the acid in water. Thus, for molybdate

$$pK^*_{a2} = [3.4(\pm 0.1)]\chi + 3.8(\pm 0.1) \quad [r^2 = 0.995] \quad (3a)$$

and for chloromethylphosphonate

$$pK^*_{a2} = [4.9(\pm 0.1)]\chi + 6.5(\pm 0.1) \quad [r^2 = 0.990] \quad (3b)$$

Using these data (for $0.2 \leq \chi \leq 0.7$) and the method of Buncel et al.,[20] the following "Brønsted" correlations can be obtained:

For molybdate

$$\log k_2(M^{-1}\ min^{-1}) = [1.8(\pm 0.1)]pK^*_{a2} - 8.6(\pm 0.8) \quad [r^2 = 0.980] \quad (4a)$$

and for chloromethylphosphonate

$$\log k_2(M^{-1}\ min^{-1}) = [0.83(\pm 0.06)]pK^*_{a2} - 7.2(\pm 0.5) \quad [r^2 = 0.985] \quad (4b)$$

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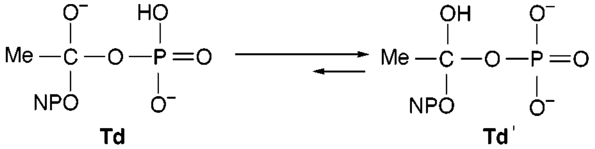
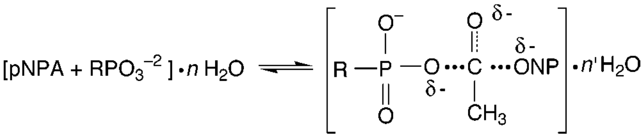
Discussion

The aqueous reaction of the phosphonates (and presumably the other oxydianions) with pNPA is, most-likely, a concerted reaction. This has been shown to be the case for the reaction of phenoxide nucleophiles with pNPA.[13,23] The evidence for a similar mechanism for acetyl transfers to phosphonates is as follows: (1) The Brønsted plot (Figure 1) is linear for the reaction of pNPA with a series of phosphonates, showing no break at $pK_a \approx 7$ (=pK_a of the *p*-nitrophenol leaving group). (2) The phosphate dianion **does not deviate** from this correlation. If a tetrahedral intermediate, **Td** (e.g., with a lifetime $> \sim 10^{-12}$ s) formed, it should tautomerize:



Based on an estimate of the pK_a difference, this tautomerization equilibrium constant is expected to be $\sim 10^4$ in favor of **Td′**, a species that is not on the reaction path. Thus, if **Td** did form as a discrete intermediate, the phosphate dianion should show a negative deviation (of ~4 log units) from the correlation based on the phosphonate dianions (for which such a tautomerization is not possible). (3) Using Guthrie's approach,[23] the energy of **Td** (relative to that of pNPA + RPO_3^{-2}) can be estimated to be ~34 kcal/mol. This $\Delta G^{\circ\prime}$ value is over 10 kcal/mol **greater** than the observed $\Delta G^{\ddagger}$ values (=23 kcal/mol for R = $ClCH_2$- and 22 kcal/mol for R = HO-) at 25 °C. These results are consistent with a concerted reaction or, at least, one that does not involve a tetrahedral intermediate.

Solvent Effect. The increase in the rate constant, with increasing DMSO concentration, for the reaction of anionic nucleophiles with pNPA is not surprising. What is unusual, however, is the magnitude of this rate increase with the dianionic nucleophiles when compared to that seen with monoanionic nucleophiles. It is reasonable to expect that the more strongly solvated in water a nucleophile is, the more destabilized, and thus more reactive, it will become when the solvent is changed to one like DMSO which does not effectively solvate anions. An admittedly simplistic estimation of the extent of this nucleophile solvation difference can be made from the linear portions in Figure 2. This is based on the following model:



n is the solvation number in the ground state (i.e., the number of water molecules in the primary solvation shell of the ester plus that of the nucleophile) and *n*′ is the solvation number in the transition state. The slope (=*m*) of a plot of log k_2 vs log[H_2O] (Figure 2) provides an estimate of the difference between these numbers (i.e., $m = n' - n$) and corresponds to the net number of water molecules which undergo reorganization as the transition state is approached. Thus, the reaction of dianions (molybdate and chloromethylphosphonate) involves significantly more solvent reorganization than does the reaction of the phenoxide monoanions (eqs 2a−c). Furthermore, the acetyl transfer to molybdate is accompanied by a somewhat greater solvent reorganization than that of the phosphonate. In pure water, the greater reactivity of molybdate than of the phosphonate must, therefore, reflect not so much the **number** of water molecules in transition but rather the **strength** of the interaction between the water molecules and the nucleophilic site from which they must be removed. In this regard it is interesting to note that the positive deviations of the dianions from the Brønsted correlation of the phosphonates (Figure 1) is inversely related to the calculated charge on the oxygen of the dianionic species. Using Gaussian 94, Kish and Viola[23] calculated the following charge densities: MoO_4^{2-}, −0.800; HVO_4^{2-}, −0.885; $HAsO_4^{2-}$, −0.892; and HPO_4^{2-}, −0.952. Thus, it is reasonable to assume that it should be more difficult to remove a water molecule from a nucleophilic site on phosphate than from the corresponding site on these other oxydianions.

It has been implicitly argued that the change in reactivity of acyl acceptors in media of various DMSO/water compositions is due just to the effect of solvent on the pK_a of the nucleophile. This allowed Buncel et al.[20] to propose a novel strategy for the construction of Brønsted plots. Thus, for the reaction of 4-cyanophenolate with pNPA in various DMSO/H_2O solvents, they obtained an excellent correlation between log k_2 and the pK_a^* in that particular solvent. Indeed, the β_{nuc} value they obtained in this way (=0.6) was the same as that obtained with several other phenoxide acyl acceptors, although it was lower than the β_{nuc} (=0.75) which they obtained in water using a series of substituted phenoxides. In our case, the "β_{nuc}" (=0.8) value for the phosphonate reaction obtained in this manner (eq 4b) is considerably larger than the value obtained in water (=0.3) with a series of substituted phosphonates (Figure 1). Clearly, factors other than just changing pK_a values influence the effect of solvent variation on reaction rates. In this regard, it is interesting to note that the pK_{a2} value of molybdate changes by 2.4 units as the solvent is changed from 1% to 90% v/v DMSO. Thus, this solvent change makes the *equilibrium constant* for monoprotonation of the molybdate dianion more favorable by a factor of 260. The same solvent change, however, makes the *rate constant* of acetyl transfer to the molybdate dianion more favorable by a factor of 5500. A qualitatively similar situation is seen with chloromethylphosphonate where the change in solvent has a greater effect on the acylation rate constant than on the pK_{a2}. This is in spite of the fact that protonation reduces the charge on the dianion by one unit while, in the transition state for acylation, the charge, although more dispersed, is probably not much changed from −2. Clearly the differential solvation (hydration) between the species in the ground state and in the transition state is one of the many factors which influence the sensitivity of a reaction to solvent variation. In enzymatic reactions the reactivity of oxydianions such as phosphate will be significantly increased if the ionic species is bound poorly solvated in a hydrophobic region (where it is protected from protonation) in the active site. We can get an estimate of this rate increase from an extrapolation of our data. A convenient way of doing this is illustrated in Figure 3. This is the same data as in Figure 2 except that the change in solvent composition is expressed as the mole fraction of DMSO (=χ). The linear dependence (in the region where $\chi \geq 0.2$) of log k_2 on the mole fraction of DMSO is given below for each of the oxydianions.

For 4-cyanophenolate

$$\log k_2(\mathrm{M}^{-1}\,\mathrm{min}^{-1}) = [2.7(\pm 0.1)]\chi - 0.47(\pm 0.05) \quad [r^2 = 0.997] \quad (5a)$$

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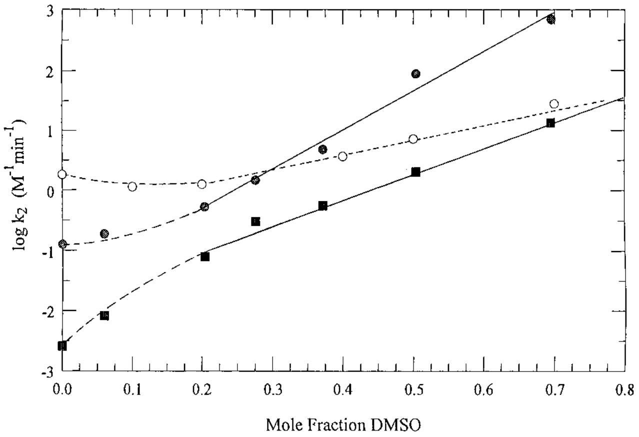

Figure 3. Replot of the data from Figure 2 in terms of the mole fraction of DMSO: (■) $ClCH_2PO_3^{2-}$, (●) MoO_4^{2-}, and (○) 4-cyanophenolate.

For chloromethylphosphonate

$$\log k_2(\mathrm{M}^{-1}\,\mathrm{min}^{-1}) = [4.3(\pm 0.2)]\chi - 1.85(\pm 0.11) \quad [r^2 = 0.992] \quad (5b)$$

For molybdate

$$\log k_2(\mathrm{M}^{-1}\,\mathrm{min}^{-1}) = [6.4(\pm 0.3)]\chi - 1.59(\pm 0.15) \quad [r^2 = 0.993] \quad (5c)$$

Table 1. Solvent Effect on Nucleophilicity

nucleophile	k_2, M^{-1} min^{-1}		$k_{\chi=1}/k_{\chi=0}$
	$\chi = 0$[a]	$\chi = 1$[b]	
4-cyanophenolate[c]	1.8	1.7×10^2	94
chloromethylphosphonate	2.5×10^{-3}	2.8×10^2	1×10^5
molybdate	1.3×10^{-1}	6.5×10^4	5×10^5

[a] Measured rate constants in water (mole fraction of DMSO = 0). [b] Calculated rate constants [eq 5] in DMSO. [c] Data from Buncel et al.[20]

This allows for easy extrapolation to pure DMSO ($\chi = 1$). These results are summarized in Table 1. It can be seen that for the chloromethylphosphonate reaction, complete stripping of the dianion of its solvating water molecules results in a rate enhancement of about 10^5-fold. Thus, for the enzymatic reaction of dianionic nucleophiles such as phosphate we might expect a significant contribution of "desolvation" to the enzymic rate enhancement. However, for monoanionic nucleophiles, such as 4-cyanophenolate, this contribution is quite modest (less than 100-fold) and for neutral nucleophiles the substitution of an aqueous solvent to a DMSO-like reaction environment is inhibitory.

Supporting Information Available: Table of log k_2 and pK_{a2}* values in solutions of various DMSO/H_2O compositions (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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