Concerted or Stepwise Mechanisms for Acyl Transfer Reactions of *p*-Nitrophenyl Acetate? Transition State Structures from Isotope Effects

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Abstract: Isotope effects have been measured for the acyl transfer reactions of *p*-nitrophenyl acetate (PNPA) with the oxyanion nucleophiles hydroxide, phenolate, and the anion of hexafluoroisopropyl alcohol; with the sulfur anions of mercaptoethanol and methyl 3-mercaptopropionate; and with the nitrogen nucleophile methoxyethylamine. The kinetic isotope effects measured were the oxygen-18 isotope effects in the carbonyl oxygen, $^{18}k_{carbonyl}$, and in the phenolic oxygen atom of the leaving group, $^{18}k_{lg}$; the β-deuterium isotope effect in the acetyl group, ^{D}k; and the nitrogen-15 isotope effect in the leaving group, ^{15}k. The equilibrium phenolic oxygen-18 ($^{18}K_{eq} = 1.0277 \pm 0.0007$) and nitrogen-15 ($^{15}K_{eq} = 1.0016 \pm 0.0006$) isotope effects for the equilibrium between *p*-nitrophenolate anion and PNPA were also determined. The ^{15}k and $^{18}k_{lg}$ kinetic isotope effects for the hydroxide reaction with PNPA are smaller than for the other oxyanion nucleophiles, suggesting an earlier transition state. For the other oxyanion nucleophiles ^{15}k was about 1.0010, versus near unity for the sulfur nucleophiles; $^{18}k_{carbonyl}$ for oxyanions ranged from 1.0039 to 1.0058; versus from 1.0117 to 1.0119 for thiolates. Values for $^{18}k_{lg}$ were between 1.0182 and 1.0210 for oxyanions, and 1.0172 and 1.0219 for thiolates. The ^{D}k effects were between 0.9481 and 0.9617 for oxyanions, and 0.9765 and 0.9780 for thiolates. The transition state structures implied by these data are consistent with studies by others using phenolate nucleophiles which concluded that the acyl transfer process is concerted, with no intermediate. The isotope effect data indicate that the transition state for the reaction with phenolate is not substantively different from that with an aliphatic oxyanion nucleophile of similar pK_a. The reactions with thiolate nucleophiles have a considerably different transition state structure, characterized by greater loss of the carbonyl π-bond and the maintenance of more positive charge on the carbonyl carbon atom. The degree of transition state bond cleavage to the leaving group is similar for oxyanion and thiolate nucleophiles. Acyl transfer to the nitrogen nucleophile methoxyethylamine gave $^{15}k = 1.0011$, $^{18}k_{lg} = 1.0330$, $^{18}k_{carbonyl} = 1.0064$, and $^{D}k = 0.9682$. These data require that bond fission to the leaving group is well advanced in the rate-limiting step.

Introduction

The occurrence of oxygen-18 exchange from solvent water into the carbonyl group of alkyl esters undergoing hydrolysis is strong evidence for a tetrahedral intermediate.[1] However, a considerable amount of evidence from recent work indicates that certain aryl esters, possessing good leaving groups, undergo acyl transfer reactions by a concerted pathway.[2-4] The switch from a tetrahedral mechanism to a concerted one could occur at a point where the tetrahedral species would have no significant lifetime and would be a transition state instead of an intermediate.

Williams and co-workers have shown that a Brønsted correlation of log k with nucleophile pK_a for the reaction of substituted phenolates with *p*-nitrophenyl acetate (PNPA) does not exhibit a break in the slope as would be expected with a stepwise mechanism.[2] In subsequent work, it was shown that there is interaction between the attacking phenolate and the departing leaving group from aryl acetates, consistent with a concerted mechanism and it was predicted that for acyl transfers between phenols and aryl acetates the concerted mechanism operates for hypothetical phenols with pK_a's between 11.7 and 2.0, where a transition takes place to a tetrahedral or acylium-like transition state, respectively.[3] Further Brønsted analysis led Jencks and co-workers to conclude that substituted phenyl formates, as well as phenyl acetates, react with phenolate anions through a concerted mechanism.[4] These conclusions have not been universally accepted, and evidence from Brønsted studies favoring a stepwise reaction pathway for aryl acetates has been presented.[5]

From application of Marcus theory, Guthrie concluded that exchange reactions of aryl esters will be concerted for phenols with pK_a values between 1 and 11, with simultaneous formation of the bond to the nucleophile and breaking of the bond to the leaving group, due to the lack of a meaningful barrier to the collapse of a tetrahedral intermediate.[6]

The concept of a tetrahedral transition state, as opposed to an intermediate, has also been proposed from theoretical studies of the attack of halides on acetyl halides.[7] From experimental gas-phase investigations of addition reactions of various nu-

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cleophiles with acyl halide substrates, Asubiojo and Brauman have concluded that the gas-phase mechanism involves a tetrahedral transition state, not an intermediate.[8] Evidence for the existence of an S_N2 pathway in solvolysis reactions of benzoyl chloride has been reported.[9]

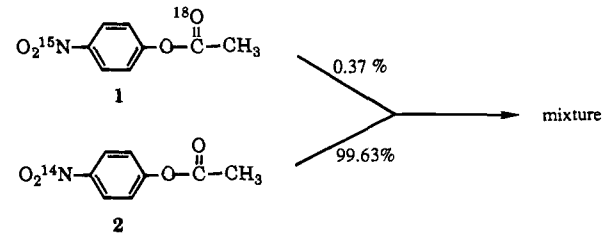
A powerful technique for learning the structure of transition states is the use of kinetic isotope effects. We report here measurements of kinetic isotope effects on the acyl transfer reactions of PNPA with various nucleophiles. With a leaving group pK_a of 7.1, this ester falls into the range of those which previous work suggests react with phenolate nucleophiles by a concerted mechanism.[2-4,6] We have measured isotope effects with phenolate as the nucleophile as well as with several nonaromatic nucleophiles of similar pK_a in order to assess any differences this change causes in transition state structure. The reaction with hydroxide was also studied. The isotope effects measured were the oxygen-18 isotope effects in the carbonyl oxygen, $^{18}k_{carbonyl}$, and in the phenolic oxygen atom of the leaving group, $^{18}k_{lg}$; the β-deuterium isotope effect in the acetyl group, ^{D}k; and the ^{15}N isotope effect in the leaving group, ^{15}k.

Kinetic isotope effects have been used by others to elucidate details of transition state structure in ester hydrolysis reactions. Direct comparison of rates were used to measure the $^{18}k_{carbonyl}$ effects on the methanolysis of phenyl benzoates.[10] The $^{18}k_{lg}$[11a] and $^{18}k_{carbonyl}$[12] have been measured for hydrolysis reactions of methyl formate. Methyl benzoate has been studied as well, where development of a remote label method allowed measurements of the isotope effects at the carbonyl carbon, the carbonyl oxygen, and the leaving group oxygen.[13]

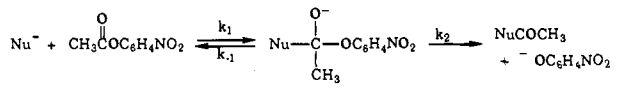
The β-deuterium isotope effect has also been used to study acyl transfer reactions.[14] This isotope effect arises from changes in the β-C−H force constant brought about by variations in hyperconjugation. As a nucleophile interacts with the carbonyl carbon, hyperconjugation is decreased as the carbonyl π-bond is lost, increasing the force constants of these C−H bonds and thus producing an inverse isotope effect ($k_H < k_D$).

The magnitudes of ^{15}k and $^{18}k_{lg}$ give a measure of the degree of bond cleavage to the leaving group. The nitrogen-15 isotope effect arises from the delocalization into the aromatic ring of the negative charge resulting from partial bond cleavage and has proven useful in the study of reactions of phosphate esters of *p*-nitrophenol.[15] To assist with the intrepretation of these data, the same isotope effects have been measured for the equilibrium between *p*-nitrophenolate anion and PNPA. We have also redetermined the equilibrium oxygen-18 isotope effect for deprotonation of *p*-nitrophenol.[16]

Scheme 1



In this work, kinetic isotope effects are reported for the acyl transfer reactions from PNPA to the nucleophiles hydroxide; the anions of phenol, hexafluoroisopropyl alcohol, mercaptoethanol, and methyl 3-mercaptopropionate; and to methoxyethylamine. The anionic nucleophiles each have pK_a's sufficiently greater than that of the leaving group so that nucleophilic attack, coupled or not with leaving group departure, will be rate-limiting. This is because if a tetrahedral mechanism is followed (eq 1), this difference in pK_a will result in $k_2 \gg k_{-1}$, and the intermediate would partition essentially completely forward to products. Therefore only the first step of a tetrahedral mechanism will exhibit isotope effects under these conditions. The major change undergone in the ester substrate in this step is the loss of the carbonyl π-bond. If a concerted mechanism is followed, then the major bonding change in the starting material should be the breaking of the bond to the leaving group.



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The isotope effect data are used to describe the transition state structures involved and to gain insight as to whether a concerted or a stepwise mechanism is followed for each reaction. A small portion of this work has been reported in a preliminary communication.[17]

The isotope effects were measured by the competitive method, using an isotope ratio mass spectrometer.[18] The oxygen-18 isotope effects and the β-deuterium isotope effects were measured by the remote label method.[13] In this method substrate is synthesized with labels at two positions, one at the site of chemical interest and the other at a position that lends itself to isolation and isotopic measurement to function as a marker. In this study the nitrogen atom in PNPA served as the remote label. For measurement of $^{18}k_{carbonyl}$, PNPA with nitrogen-15 in the nitro group and oxygen-18 in the carbonyl oxygen atom was synthesized (**1**), as was the corresponding nitrogen-14 compound (**2**) (Scheme 1). These were then mixed in proportion to reconstitute close to the natural abundance of 0.37% nitrogen-15, as the isotope ratio measurements are most accurate when samples approximate natural abundance.[18] Similarly, the $^{18}k_{lg}$ values were measured using the compound with nitrogen-15 in the nitro group and with oxygen-18 in the phenolic oxygen atom (**3**). The ^{D}k effects were measured using the compound with three deuteriums in the acyl methyl group and with nitrogen-15 in the nitro group (**4**). No synthesis is necessary in order to measure the ^{15}k effects, as these are measured using the natural abundance compound.

The measurement of heavy atom isotope effects using an isotope ratio mass spectrometer is a much more sensitive and accurate method than direct comparison of rates or of spectroscopic changes, due to the small magnitudes of the effects.

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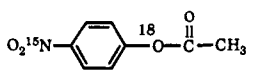

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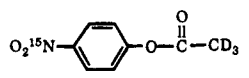

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Experimental Section

Natural abundance PNPA was purchased from Aldrich and recrystallized from hexanes before use. [^{18}O]Water, $NH_4{}^{14}NO_3$, and $NH_4{}^{15}NO_3$ were purchased from Isotec. Acetyl-d_3 chloride and $FeCl_3$ were purchased from Aldrich. 4-(Dimethylamino)pyridine (from Aldrich) was dried under vacuum in a desiccator over P_2O_5. Chloroform was dried over P_2O_5 and distilled. Aqueous solutions of PNPA were made by dissolving the compound in a small amount (2−3 mL) of methylene chloride, adding water, and then removing the organic solvent by rotary evaporation at room temperature and filtering the resulting solution. This gave aqueous solutions with concentrations of 2−3 mM PNPA. Solutions of similar concentrations could also be made by prolonged stirring of PNPA in water, but slight hydrolysis occurred during the long time required.

Preparation of [^{14}N]PNPA (2). This was prepared from the sodium salt of [^{14}N]-*p*-nitrophenol[19] using a variation of the method of Chattaway.[20] Four grams of the nitrophenolate salt (24.8 mmol) was added to 70 mL of water with 1.3 g of sodium hydroxide and about 20 g of ice. Acetic anhydride (4 mL) was added, and the mixture was shaken in a separatory funnel for 2 min. The aqueous mixture was extracted with methylene chloride (100 mL), and the organic fraction was washed once with water and then dried over magnesium sulfate. The solvent was removed by rotary evaporation, and further drying under vacuum removed most of the remaining residual acetic anhydride and acetic acid. Recrystallization from hexanes gave the pure product (3.5 g, 19.3 mmol, 78%).

Preparation of [^{15}N,*phenolic*-^{18}O]PNPA (3). [^{15}N]-*p*-Nitrophenol was prepared by nitrating triphenyl phosphate with [^{15}N]ammonium nitrate followed by hydrolysis using a method similar to that previously described for the preparation of the [^{14}N]phenolate.[19] The phenolic oxygen atom of the sodium salt thus obtained was exchanged in [^{18}O]water under basic conditions.[21] After recovery of labeled water by distillation, water was added and the neutral [^{15}N,^{18}O]-*p*-nitrophenol was isolated by acidification followed by extraction with ether. The ether extracts were dried over magnesium sulfate, and the solvent was removed by rotary evaporation. After drying over P_2O_5, the [^{15}N,^{18}O]-*p*-nitrophenol (57 mg, 0.4 mmol) was dissolved in 2 mL of chloroform followed by 2 equiv of acetic anhydride and 1 equiv of 4-(dimethylamino)pyridine, and the reaction was stirred at 50 °C for 1 h. After cooling to room temperature, the reaction mixture was concentrated by rotary evaporation and chromatographed using flash silica gel, eluting with a mixture of equal parts methylene chloride and cyclohexane. After recrystallization from hexanes, the product (51 mg, 0.28 mmol, 70%) was analyzed by mass spectroscopy and found to consist of 86.5% of the ^{15}N,^{18}O compound; 11% ^{15}N,^{16}O; and 2.5% ^{14}N,^{18}O.

Preparation of [^{15}N,*carbonyl*-^{18}O]PNPA (1). [^{18}O]Acetyl chloride was prepared using the method of Hill and reacted in situ.[22] Commercial "anhydrous" $FeCl_3$ (500 mg, 3.1 mmol) was dried overnight by refluxing in 25 mL of carbon tetrachloride under a stream of dry nitrogen gas.[22] After cooling, the carbon tetrachloride was removed by distillation at 30 °C under reduced pressure (water aspirator) and replaced with 3 mL of dry chloroform. Then 1,1,1-trichloroethane (360 μL, 3.6 mmol) was added, followed by [^{15}N]-*p*-nitrophenol (100 mg, 0.7 mmol) and 55 μL (2.75 mmol) of [^{18}O]water in that order. The reaction mixture was vigorously stirred at 42 °C. After 90 min, the mixture was cooled to room temperature and filtered, and the product was isolated by chromatography and recrystallization as described for the phenolic-^{18}O-labeled compound. The purified product (70 mg, 0.38 mmol, 54% based on the limiting reagent [^{15}N]-*p*-nitrophenol) was analyzed by mass spectrometry and found to consist of 76.4% of the ^{15}N,^{18}O compound; 20.3% ^{15}N,^{16}O; and 3.3% ^{14}N,^{18}O.

Preparation of [β-D$_3$,^{15}N]PNPA (4). To 3 mL of chloroform was added 100 mg (0.7 mmol) of [^{15}N]-*p*-nitrophenol, followed by 61 μL (0.86 mmol) of acetyl-d_3 chloride and then 87.8 mg (0.7 mmol) of 4-(dimethylamino)pyridine. After 3 h at room temperature, the mixture was partitioned between 0.05 N HCl and methylene chloride. The aqueous layer was washed again with methylene chloride, the combined organic layers were dried over magnesium sulfate, and the solvent was removed by rotary evaporation. The product was purified by the same methods as for the other isotopic versions of this compound. Analysis by mass spectroscopy showed it to consist of 97% ^{15}N−D$_3$ compound, with the remainder ^{15}N−D$_2$.

Kinetic Isotope Effect Determinations. General Methods. Reactions were performed using 40−50 mL of PNPA solution, to which was added buffer and, except for the hydroxide reactions, the appropriate nucleophile. After partial completion, reactions were stopped by extraction with 70 mL of methylene chloride. At the pH's where these extractions were performed, control experiments verified that the *p*-nitrophenol produced by reaction remained deprotonated in the aqueous layer, while the remaining PNPA substrate was quantitatively extracted into the organic layer. The amount of *p*-nitrophenol present in the aqueous layer was assayed by measuring the absorbance at 400 nm of an aliquot added to dilute NaOH. The *p*-nitrophenol product was then isolated from the aqueous layer by acidification to pH 4 and three extractions with ether. The ether layers were combined and dried over magnesium sulfate, the ether was removed by rotary evaporation, and the *p*-nitrophenol was then purified by vacuum sublimation at 90 °C. The *p*-nitrophenol was transferred to a quartz tube for combustion, and the nitrogen produced was then analyzed for isotopic composition.

The residual substrate, which was in the methylene chloride layer, was concentrated to a small volume (approximately 3 mL) by rotary evaporation, and then 25 mL of water was added followed by 1 mL of 5 N NaOH solution. The remaining methylene chloride was then removed by rotary evaporation, and the resulting aqueous solution was allowed to stand at room temperature for an additional 15 min. PNPA hydrolysis under these conditions is virtually instantaneous. The *p*-nitrophenol released in this way from the residual substrate was assayed from the absorbance at 400 nm of an aliquot added to dilute NaOH. The *p*-nitrophenol was then isolated and prepared for isotopic analysis as previously described. The relative amounts of *p*-nitrophenol present after partial reaction and that released from the residual substrate were used to calculate the fraction of reaction *f*, used in calculations of the isotope effects. Control experiments verified that isolation of *p*-nitrophenol from product and from residual substrate samples was quantitative.

The isotopic composition of the starting compounds was determined in two ways, by combustion of the PNPA itself and by subjecting samples to complete reaction and analyzing the *p*-nitrophenol product. This serves as a control to verify that no artifacts or isotopic fractionations are introduced by any of the experimental procedures involved. The isotopic ratios obtained by the two methods were the same within experimental error.

Conditions for Isotope Effect Reactions. The rates for each reaction were found to be independent of buffer concentration, ruling out general base assistance by the buffer. Analysis of reaction mixtures by NMR showed that the product formed in each reaction was that expected from direct nucleophilic attack and that no acetate was formed from general base-catalyzed attack of water. Concentrations of PNPA were 2−3 mM in all reactions. Descriptions of the specific reaction conditions used for each reaction follow.

Hydroxide Reaction. Reactions were run at room temperature, with 100 mM CHES buffer, pH 9.5. The half-life for this reaction was approximately 2 h.

Reaction with Phenol. In order to minimize the light-catalyzed air oxidation of phenol which occurs in basic solution, these reactions were carried out in a darkened room, in stoppered flasks cooled in an ice bath. Reactions were run at a phenol concentration of 17 mM with 50 mM CHES buffer at a pH of 9.7, where the half-life was about 10 min. The extracted *p*-nitrophenol samples contained phenol. Most of this was removed by extracting aqueous solutions of the *p*-nitrophenol samples at pH 11.5 with ether. The aqueous layers were then acidified, and *p*-nitrophenol was isolated as usual. Traces of phenol remained

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and were carried through the sublimation process with the *p*-nitrophenol samples, but this did not interfere with the isotopic analysis of nitrogen.[23]

Reaction with Hexafluoroisopropyl Alcohol. Reactions were run at room temperature at a hexafluoroisopropyl alcohol concentration of 8.3 mM, with 55 mM CHES buffer, pH 9.0. The half-life for the reaction was about 3 min.

Reaction with Mercaptoethanol. Reactions were run at room temperature, at mercaptoethanol concentrations of 1.65 mM, with 55 mM CHES buffer, pH 9. The half-life for reaction was about 10 min.

Reaction with Methyl 3-Mercaptopropionate. Reactions were run at room temperature, at methyl 3-mercaptopropionate concentrations of 1.5 mM, with 55 mM CHES buffer, pH 9. The reaction had a half-life of about 5 min.

Reaction with 2-Methoxyethylamine. A stock solution of 0.2 M 2-methoxyethylamine was titrated to pH 9 with dilute HCl. This stock was used to make reaction mixtures up to a concentration of 6 mM of 2-methoxyethylamine, with 55 mM CHES buffer, pH 9. The reaction had a half-life of about 5 min at room temperature.

Equilibrium Isotope Effect Determinations. Equilibrium of *p*-Nitrophenolate Anion between Water and Chloroform. Fifteen milligrams of *p*-nitrophenol was dissolved in 25 mL of water and titrated to pH 9.5 using an aqueous solution of tetra-*n*-butylammonium hydroxide. Chloroform (25 mL) was added, and the mixture was stirred vigorously for 3 days in a stoppered Erlenmeyer flask. The aqueous and chloroform layers were then separated; assays showed the *p*-nitrophenolate to be about equally partitioned between the two phases. *p*-Nitrophenol was isolated from the aqueous layer by acidification to pH 4 and extraction with ether. The chloroform layer was added to 25 mL of water and the chloroform removed by rotary evaporation, followed by acidification and extraction with ether. The *p*-nitrophenol obtained from each phase was further purified by sublimation before combustion and isotopic analysis.

Equilibrium of *p*-Nitrophenolate Anion and *p*-Nitrophenyl-acetate. *p*-Nitrophenol (20 mg, 0.14 mmol) was dissolved in 20 mL of water and titrated to pH 9.5 with aqueous tetra-*n*-butylammonium hydroxide. The solution was dried by rotary evaporation and then under vacuum over P_2O_5. The resulting tetra-*n*-butylammonium salt of *p*-nitrophenol was dissolved in 3 mL of chloroform, transferred to a pressure tube fitted with a threaded teflon cap and an O-ring seal, and treated with 4-acetoxybenzaldehyde (150 μL, 1.07 mmol). The tube was sealed and stirred at 40 °C. Assays showed that the reaction comes to chemical equilibrium within an hour, with about 40% of the initial *p*-nitrophenolate converted to PNPA. To assure that isotopic equilibrium was reached, reactions were run for 24, 48, and 72 h. Reactions were quenched by addition of the chloroform mixture to 50 mL of water containing 175 μL of 1 N HCl followed by shaking, which caused discharge of the yellow color of *p*-nitrophenolate, indicating that all of the free phenol was protonated. The chloroform was removed by rotary evaporation, and the aqueous material was extracted three times with 30 mL of ether. This step isolated both of the acetate esters and both phenols in the ether layer, leaving tetra-*n*-butylammonium salts in the aqueous phase, which was found necessary to facilitate subsequent separations. The ether layers were concentrated by rotary evaporation, and the residue was partitioned between 25 mL of cold CHES buffer (250 mM, pH 9.5) and 50 mL of chloroform. The chloroform layer contained both acetate esters, while *p*-nitrophenol and *p*-hydroxybenzaldehyde remained in the buffered aqueous layer. Assays of both fractions showed the same ratio of *p*-nitrophenol to PNPA as as was present in the reaction mixtures before they were quenched, indicating that the isolation procedures had not caused further reaction or disturbed the equilibrium. The mixed *p*-nitrophenol and *p*-hydroxybenzaldehyde were isolated from the aqueous layer by acidification and ether extraction. Since the phenols were found to be easier to separate from one another than the respective acetate esters, the esters were hydrolyzed by adding the chloroform layer to 25 mL of water containing 3 mL of 5 N NaOH, and subjected to rotary evaporation to remove the chloroform. After standing for 1 h, the resulting solution was acidified and extracted with ether. The ether solutions were dried over magnesium sulfate and concentrated by rotary evaporation. The *p*-nitrophenol was separated from *p*-hydroxybenzaldehyde by column chromatography on flash silica gel, eluting with methylene chloride. The *p*-nitrophenol eluted far ahead of the other phenol and was further purified by sublimation before combustion and isotopic analysis. Since *p*-hydroxybenzaldehyde contains no nitrogen atoms, its presence would not interfere with the isotopic analysis. However, its presence in large excess in the samples would have required a large sample size for combustion in order to produce enough molecular nitrogen, which would have led to rupture of the quartz tubes used for combustion from the large amounts of carbon dioxide produced.

As a control, in a separate experiment this equilibrium was started from the reverse direction. In this case *p*-hydroxybenzaldehyde (25 mg, 0.205 mmol) was dissolved in 25 mL of water and titrated to pH 10 with aqueous tetra-*n*-butylammonium hydroxide. The solution was dried by rotary evaporation and then under vacuum over P_2O_5. The tetra-*n*-butylammonium salt was taken up in 3 mL of chloroform, transferred to the pressure tube containing PNPA (75 mg, 0.414 mmol), sealed, and stirred at 40 °C. An aliquot was removed for isotopic analysis after 1, 4, and 6 days. Samples were prepared for isotopic analysis as described for the first method.

Equilibration of PNPA between Water and Chloroform. PNPA (50 mg) was dissolved in 4 mL of chloroform and added to 200 mL of water with 15 μL of 20% acetic acid. The slight acidification of the water layer minimized spontaneous hydrolysis of the ester during the experiment. The mixture was stirred vigorously for at least 48 h. After the aqueous and organic phases were separated, the PNPA in the aqueous layer was extracted with 75 mL of chloroform. An aliquot of each chloroform layer was dried with magnesium sulfate and concentrated by rotary evaporation, and the PNPA was transferred to quartz tubes for combustion.

Deprotonation of *p*-Nitrophenol. The equilibrium phenolic oxygen-18 isotope effect for deprotonation of *p*-nitrophenol was measured using the remote labeled compound [^{15}N,^{18}O]-*p*-nitrophenol, using the procedure previously described for determination of the equilibrium ^{15}N isotope effect.[15a]

Isolation of Molecular Nitrogen for Isotopic Analysis. Combustion was used to convert the nitrogen in samples to be analyzed to N_2, which was isolated for isotope ratio analysis using procedures previously described.[15b] The isotope ratio mass spectrometer measures the ^{15}N to ^{14}N ratio of a sample relative to that of a standard sample of nitrogen gas.[18]

Data Analysis. The kinetic isotope effects were calculated using the isotopic ratios from the product at partial reaction (R_p), from the remaining substrate (R_s), and from the isotopic ratio in the starting material (R_0). Equation 2 was used to calculate the observed isotope effect from the isotopic ratios of the product and starting material at known fractions of reaction, *f*. Equation 3 was used to calculate the observed isotope effect from the isotopic ratios of the residual substrate and the starting material.

$$\text{isotope effect} = \log(1-f)/\log(1-f(R_p/R_0)) \quad (2)$$

$$\text{isotope effect} = \log(1-f)/\log[(1-f)(R_s/R_0)] \quad (3)$$

The equilibrium isotope effects were calculated as the ratio of the isotopic ratios between the two equilibrated species.

The oxygen-18 and the β-deuterium isotope effects were measured using the mixed double-labeled substrates. These experiments yield an observed isotope effect which is the product of the effect due to nitrogen-15 substitution and that due to the oxygen-18 or the three deuteriums. The observed effects from these experiments were corrected for the nitrogen-15 effect and for incomplete levels of isotopic incorporation in the starting material.[24] Further descriptions of these methods and of the apparatus used have been published elsewhere.[18]

Results

The kinetic isotope effect results are tabulated in Table 1. The oxygen-18 and ^{D}k isotope effects have been corrected for

(23) If most of the phenol is not removed prior to combustion, the large amounts of CO_2 produced can cause the quartz tubes to shatter.

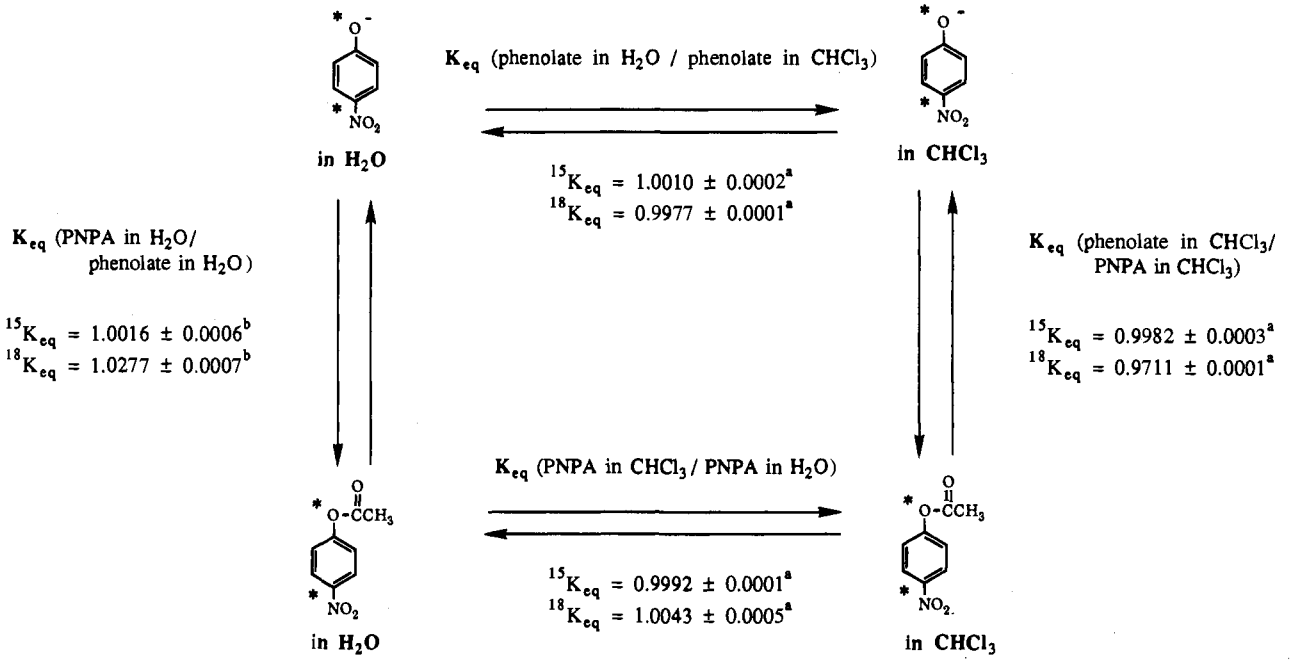
(24) Caldwell, S. R.; Raushel, F. M.; Weiss, P. M.; Cleland, W. W. *Biochemistry* **1991**, *30*, 7444−7450.

Table 1. Isotope Effects for Acyl Transfer Reactions of *p*-Nitrophenyl Acetate with Various Nucleophiles[a]

nucleophile (p*K*)	^{15}k (ratio)[b]	$^{18}k_{lg}$ (ratio)[c]	$^{18}k_{carbonyl}$	^{D}k[f]
hydroxide	1.0002 ± 0.0001	1.0135 ± 0.0007	1.0039 ± 0.0003	0.9562 ± 0.0008
(15.7)	(0.13)	(0.49)		
phenolate	1.0010 ± 0.0002[d]	1.0199 ± 0.0009[d]	1.0043 ± 0.0008[b]	0.9583 ± 0.0010[b]
(9.9)	(0.63)	(0.72)		
	1.0009 ± 0.0002[e]	1.0182 ± 0.0009[e]	1.0039 ± 0.0008[c]	0.9617 ± 0.0010[c]
	(0.56)	(0.66)		
$(CF_3)_2CHO^-$	1.0010 ± 0.0002	1.0210 ± 0.0010	1.0058 ± 0.0006	0.9481 ± 0.0030
(9.3)	(0.63)	(0.76)		
anion of mercaptoethanol	1.0001 ± 0.0003	1.0219 ± 0.0009	1.0119 ± 0.0003	0.9780 ± 0.0008
(9.5)	(0.06)	(0.79)		
anion of $HSCH_2CH_2CO_2CH_3$	1.0003 ± 0.0001 (0.19)	1.0172 ± 0.0004	1.0117 ± 0.0004	0.9765 ± 0.0006
(9.3)	(0.19)	(0.62)		
methoxyethylamine	1.0011 ± 0.0001	1.0330 ± 0.0007	1.0064 ± 0.0003	0.9682 ± 0.0010
(9.7)	(0.69)	(1.19)		

[a] Reactions were all carried out at 25 °C except for that with phenolate. [b] Ratios in parentheses are those of the kinetic effect to the $^{15}K_{eq}$ effect of 1.0016. [c] Ratios in parentheses are those of the kinetic effect to the $^{18}K_{eq}$ effect of 1.0277. [d] Experiments at 0 °C. [e] Corrected to 25 °C. [f] Isotope effect for three deuterium atoms.

Scheme 2



[a]Experimental value. [b]Calculated value.
*Indicates sites of equilibrium isotope effect measurements.

the ^{15}k effect and for isotopic incorporation. At least six experiments were run for each set of conditions to varying fractions of reaction, which yielded 12 determinations for each isotope effect. The effects calculated from R_p and R_0 and those from R_s and R_0 gave excellent agreement in all cases and were averaged together to give the mean values recorded in the table, along with the standard errors. The data for the phenolate reactions, which were run at a lower temperature than the others, have been corrected for this difference. An approximation of the temperature effect on the isotope effects was made using the equation ln(IE at 25 °C) = (273 K/298 K) × ln(IE at 0 °C).

The ^{D}k isotope effects measured in this work for the acyl transfer from PNPA to hydroxide and to phenolate (Table 1) are slightly more inverse than the figures of 0.970 ± 0.009 and 0.971 ± 0.020 previously determined by direct spectroscopic comparison for these reactions.[14c]

The equilibrium isotope effect results are shown in Scheme 2. The isotope effects on the equilibria illustrated at the upper, lower, and right-hand sides of Scheme 2 were determined experimentally. Since the product of the equilibrium isotope effects for the four legs of the thermodynamic box must equal unity, the three experimental numbers were used to calculate the isotope effect for the equilibrium between *p*-nitrophenolate and PNPA in water. This indirect approach was necessary because significant hydrolysis of PNPA occurs over the time period necessary to establish the desired equilibrium in water.

The $^{18}K_{eq}$ isotope effect for the equilibrium deprotonation of *p*-nitrophenol determined in this work was found to be 1.0153 ± 0.002, comparable to the value of 1.018 ± 0.002 previously reported.[16]

Discussion

The use of kinetic isotope effects is an effective method for ascertaining transition state structure. Accurate interpretation of isotope effects in terms of fractional bonding changes on going from starting materials to transition states depends on the proper choice of a yardstick for comparison with measured

isotope effects, which can come from computational studies or from isotope effects measured on model systems whose transition state structures are already known from other methods.

The $^{18}k_{carbonyl}$ and ^{D}k effects expected for a carbonyl addition reaction have been studied computationally and were found to vary as nearly linear functions of the extent of bond formation between the nucleophile and the carbonyl carbon.[14a] The computed carbonyl oxygen isotope effects were normal, a consequence of the conversion of the carbonyl double bond of the reactant to the more nearly single bond of the assumed tetrahedral intermediate, and were found to reach a maximum value of about 1.025 for a very late transition state.[14a] For comparison, the *equilibrium* isotope effect for formation of a tetrahedral intermediate has been estimated at 1.03.[13] The largest experimental $^{18}k_{carbonyl}$ effect reported to date is 1.024, measured for the methanolysis of phenyl benzoate.[10]

The computed ^{D}k values were inverse and were estimated to reach a value of about 0.89 for a very late transition state.[14a] This compares well with experimental results which were used to arrive at an estimated value of 0.87 for the equilibrium isotope effect for formation of a tetrahedral intermdiate.[14a] Both $^{18}k_{carbonyl}$ and ^{D}k reflect changes in the carbonyl group and in the partial positive charge on the carbonyl carbon resulting from nucleophilic attack. One may reasonably make different predictions about the magnitudes of such changes for a stepwise versus a concerted mechanism. In the reactions studied in this work, due to the differences in pK_a's between the anionic nucleophiles and leaving group, any tetrahedral intermediate would partition essentially completely forward to products. In this case the isotope effects measured would be the kinetic effects on formation of the intermediate. Such an intermediate is of low stability, and it is therefore thought, on the basis of the Hammond postulate and related ideas, that the transition state occurring just before it should resemble the intermediate more than the reactant.[25] On this basis one might reasonably expect to observe isotope effects indicating significant changes in the carbonyl π-bond, whereas in a concerted mechanism this bond may or may not be altered, depending upon whether the transition state is an associative one.

Changes in bonding to the *p*-nitrophenol leaving group are measured by $^{18}k_{lg}$ and ^{15}k. Possible yardsticks for interpreting the magnitudes of these isotope effects are the corresponding equilibrium isotope effects on the deprotonation of *p*-nitrophenol, which are $^{18}K_{eq} = 1.0153$ and $^{15}K_{eq} = 1.0023$.[15a] The nitrogen-15 isotope effect arises from delocalization of negative charge into the aromatic ring, reflecting the contribution of the quinonoid resonance structure **5**. However, deprotonation involves breaking a bond to a proton, not to a carbonyl group. One indication that these are not equivalent is the finding that an aryl group in an acetate ester has an effective charge of +0.7 relative to the neutral phenol as measured from Brønsted correlations, a difference which reflects the greater electron withdrawing character of the acyl group.[26] To assist in interpreting the magnitudes of the leaving group isotope effects, we measured the equilibrium isotope effects at the same atoms (indicated by asterisks in eq 4a) for the equilibrium between *p*-nitrophenolate and PNPA. The direct equilibration of [^{15}N,^{18}O]-*p*-nitrophenol with [^{14}N]PNPA was not practical since samples must approximate natural abundance for the most accurate isotopic analysis. By setting up a reaction between appropriate concentrations of the anion of *p*-nitrophenol and another acetate ester having a leaving group of similar pK_a, it is possible to attain an equilibrium where *p*-nitrophenol is partitioned between these two species according to eq 4b below. The acetate ester of *p*-hydroxybenzaldehyde was found to work well for this purpose.

$$^{-}OC_6H_4NO_2 + CH_3COC_6H_4CHO \rightleftharpoons {}^{-}OC_6H_4CHO + CH_3COC_6H_4NO_2 \quad \text{4b}$$

When this experiment was attempted in aqueous solution, it was found that at a pH necessary to keep the phenols deprotonated so that reaction could occur, competitive aqueous hydrolysis of PNPA occurred. Therefore the equilibrium isotope effects were measured in anhydrous organic solution, and corrections were made for the effects of the solvent. The thermodynamic box in Scheme 2 illustrates how this problem was solved. First, the tetra-*n*-butylammonium salt of *p*-nitrophenol was equilibrated between water and chloroform. There are isotope effects on this equilibrium which arise from a difference in the degree of charge delocalization in the anion in the two solvents. Evidence for this can also be seen in the spectral difference between phenolate anion in aqueous solution, where λ_{max} is 400 nm and the extinction coefficient is 18.3 mM^{-1} cm^{-1}, and in chloroform, where these are 425 nm and 24.2 mM^{-1} cm^{-1}, respectively. The difference is most easily explained as arising from greater charge delocalization in the nonpolar chloroform solvent, leading to a larger contribution of the quinonoid resonance structure **5**. The isotope effects on partitioning between the solvents are in the direction expected from this line of reasoning. In the phenolic oxygen atom, oxygen-18 enriches in the chloroform phase where the quinonoid resonance contribution is enhanced, since in this structure the phenolic oxygen atom has a higher bond order. In the nitro group, the bond order between nitrogen and oxygen decreases and that between nitrogen and carbon increases in the quinonoid structure. Since N−O bonds are more stiffening than N−C bonds, ^{15}N will enrich in the aqueous phase, where the quinonoid contribution is smaller.

The isotope effects were then measured for the equilibrium between phenolate anion and PNPA in chloroform. As a check, the equilibrium was approached from both directions, by starting with *p*-nitrophenolate and 4-acetoxybenzaldehyde and, in separate experiments, by starting with the anion of *p*-hydroxybenzaldehyde and PNPA. The differences between the equilibrium isotope effects measured using each method were less than the standard errors. In order to complete the thermodynamic box, the isotope effects for partitioning PNPA between water and chloroform were measured. The magnitudes of these effects are similar to those measured for the partitioning of the nitrophenolate anion, although the spectral difference between the two solutions is much smaller for PNPA. There is no significant difference in the λ_{max} of 269 nm, although the extinction coefficient is larger in chloroform, 15.0 mM^{-1} cm^{-1}, versus 13.6 mM^{-1} cm^{-1} in water.

The isotope effects for these three sides of the thermodynamic box were used to calculate the expected equilibrium effects for *p*-nitrophenolate versus PNPA in aqueous solution, $^{15}K_{eq} = 1.0016 \pm 0.0006$ and $^{18}K_{eq} = 1.0277 \pm 0.0007$.

For comparison, the kinetic isotope effects measured for the aqueous hydrolysis of the dianion of *p*-nitrophenyl phosphate are $^{15}k = 1.0028$ and $^{18}k_{lg} = 1.0189$.[15b] This reaction is known from Brønsted studies to be concerted with a very dissociative

(25) Thornton, E. K.; Thornton, E. R. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum Press: New York, 1978; Chapter 1.

(26) Deacon, T.; Farrar, C. R.; Sikkel, B. J.; Williams, A. *J. Am. Chem. Soc.* **1978**, *100*, 2525.

transition state, with the bond to the *p*-nitrophenol leaving group nearly completely broken.[27] Thus these effects should represent essentially complete bond cleavage to the leaving group for a phosphate monoester. It is logical that the maximal oxygen-18 effect representing complete bond cleavage for an acetate ester should be larger than that for a phosphate ester because an acyl group has greater electron withdrawing character than a phosphoryl group, which would be expected to result in a higher bond order between the leaving group oxygen atom and an ester carbonyl group, than between the leaving group oxygen atom and a phosphoryl group. A measure of this difference in electron withdrawing character can be seen in measurements from Brønsted correlations that show an aryl group in an acetate ester has an effective charge of +0.7 relative to a neutral phenol, as compared with +0.36 for a phosphate monoester.[26,28]

The isotope effects in the *p*-nitrophenyl leaving group are complicated by the fact that as the bond to the leaving group is partially broken in the transition state, delocalization will result in some increase in C−O bond order within the *p*-nitrophenol from contribution of resonance structure **5**, compensating for some of the loss of the bond order to the carbonyl carbon atom. This will lower the magnitude of the isotope effect which would otherwise arise from loss of this leaving group bond compared to that expected for an alkyl leaving group. Evidence for such transition state delocalization, as well as a measure of its extent, is seen in the magnitudes of the ^{15}N isotope effects. In the transition state for the phosphoryl monoester reaction, the departing phenolate anion is in close proximity to the negatively charged phosphoryl group. Charge repulsion will likely increase charge delocalization via contribution of the quinonoid resonance form, increasing ^{15}k and decreasing ^{18}k for the phosphoryl reaction. This effect will not be present in the transition state for acyl transfer. This illustrates the importance of a proper choice for calibrating these leaving group isotope effects. The experimentally determined equilibrium effects are the best choice for this purpose. In Table 1 for each reaction the ratios of the kinetic effects ^{15}k and $^{18}k_{lg}$ to the corresponding equilibrium effects $^{15}K_{eq}$ and $^{18}K_{eq}$ are given in parentheses. While exact fractions of bond cleavage cannot be determined, they can be approximated, and comparisons between the acyl transfer reactions studied with different nucleophiles can still be confidently made.

Consideration must be given to expected magnitudes of leaving group isotope effects if a tetrahedral mechanism is followed. In the reactions with oxyanion and thiolate nucleophiles, the observed isotope effects would be those on the first step, formation of the tetrahedral intermediate. In esters, the bond between the carbonyl carbon and the oxygen of the leaving group is stronger than an ordinary single bond. Since the C−O *p*-nitrophenol bond will be weaker in a tetrahedral intermediate than in PNPA, small isotope effects in the leaving group are expected on formation of an intermediate. Estimates using Pauling's rule give a bond order of approximately 1.19 for this bond, similar to that of 1.15 for the C−O bond in methyl formate. The $^{18}k_{lg}$ isotope effect for the alkaline hydrolysis of methyl formate, where formation of a tetrahedral intermediate is rate-limiting, is 1.009; for the hydrazinolysis reaction where breakdown of the intermediate is rate-determining, $^{18}k_{lg}$ is 1.062.[11a] Thus for methyl formate, $^{18}k_{lg}$ for formation of a tetrahedral intermediate was about one-seventh as large as that seen for a step in which the bond to the leaving group was breaking. A similar ratio has been observed for reactions of methyl benzoate.[13] Using the $^{18}K_{eq}$ value of 1.0277 as an approximation of the upper limit for the isotope effect expected for leaving group departure, one would then expect $^{18}k_{lg}$ to be approximately 1.0040 or smaller for a tetrahedral mechanism.

Oxyanion Nucleophiles. The isotope effect data for the oxyanion nucleophiles (Table 1) indicate that considerable bond cleavage to the leaving group has occurred in the transition state, especially for the anions of phenol and of hexafluoroisopropyl alcohol. On the basis of comparisons of the magnitudes of the experimental isotope effects with their expected values for achieving a tetrahedral intermediate or for bond cleavage to the leaving group, the data argue that the bond to the leaving group is weakened significantly more than expected for a tetrahedral mechanism, while the carbonyl π-bond is much less affected. These observations are more consistent with a transition state for a concerted displacement than that for formation of a tetrahedral intermediate. The ratios of the ^{15}k and $^{18}k_{lg}$ leaving group effects to their equilibrium values are fairly similar to one another except for the hydroxide reaction. The smaller ratio for ^{15}k in this reaction indicates that little if any of the charge arising from partial cleavage of the bond to the leaving group is delocalized into the aromatic ring at the transition state, in contrast to the other oxyanion nucleophiles. It can also be noted that while the leaving group isotope effects are smaller for the hydroxide reaction than for the other two oxyanion nucleophiles, the $^{18}k_{carbonyl}$ and ^{D}k effects are more similar between all three nucleophiles. This likely reflects an earlier transition state with respect to leaving group departure for the stronger nucleophile hydroxide, but with a similar degree of change in the acyl group at the transition state for all three oxyanion nucleophiles.

The previously cited Brønsted studies which have supported concerted acyl transfer for aryl acetates have utilized phenolate nucleophiles.[2-4] We chose the anion of hexafluoroisopropyl alcohol to serve as an aliphatic oxyanion nucleophile with a pK_a similar to that of phenol in order to determine if this difference in nucleophile property had any effect on transition state structure. The data obtained indicate a very similar transition state structure for these two nucleophiles. This indicates that the pK_a of oxyanion nucleophiles is the determining factor for transition state structure and that aromaticity and the accompanying delocalized nature of the attacking charge do not result in significant alterations in transition state structure.

Thiolate Nucleophiles. Comparisons of the data for these two nucleophiles with those of the oxyanions reveal some interesting differences. The $^{18}k_{lg}$ values are similar for the two classes of nucleophile, but the ^{15}k effects are essentially unity for both of the thiolate reactions. This suggests that even though the bond to the leaving group is broken to a similar degree as in the oxyanion reactions, in those with thiolates the developing charge on the leaving group has not delocalized into the aromatic ring. The magnitudes of the $^{18}k_{carbonyl}$ effects with thiolate nucleophiles are twice those of the oxyanion reactions, indicating greater loss of the carbonyl π-bond. By contrast, the ^{D}k effects are only about half as inverse for the thiolate reactions. This indicates that more of the carbonyl π-bond has been lost, and the residual positive charge on the carbonyl carbon atom leads to less loss of hyperconjugation than in the corresponding oxyanion reactions. The data as a whole indicate a transition state structure that resembles **6**, in which the carbonyl carbon atom maintains more of its ground state partial positive charge as a result of the greater loss of the carbonyl π-bond. The highly polarizable sulfur atom may be nucleophilically effective at adding to the carbonyl bond while not diminishing the electropositivity of the carbonyl carbon atom as much as the oxyanion nucleophiles. Maintaining such positive character at

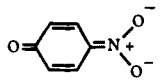
(27) Benkovic, S. J.; Schray, K. J. The Mechanism of Phosphoryl Transfer. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum Press: New York, 1978; Chapter 13.

(28) Bourne, N.; Williams, A. *J. Org. Chem.* **1984**, *49*, 1200−1204.

the carbonyl carbon would explain the hyperconjugation of the β-hydrogen atoms, as well as the lack of delocalization of the developing electron density on the leaving group, since proximity to the partial positive charge on the carbonyl carbon will favor this electron density remaining on the phenolic oxygen atom of the departing leaving group. Since the thiolate anions used have similar pK_a's to the phenolate and hexafluoroisopropyl alcohol anions, the differences in transition state structure observed here arise from differences in the nucleophilic properties of sulfur versus oxygen.

It is interesting that Brønsted-type correlations of log k with the pK_a's of thiol anions for the acyl transfer reactions with PNPA and other phenyl acetates exhibit the breaks in slope, where the pK_a's of nucleophile and leaving group are equal, that have usually been interpreted to result from a change in rate-limiting step in a tetrahedral mechanism.[29] It is the lack of such behavior with phenolate nucleophiles that has been cited as evidence that acyl transfers between phenolates are concerted.[2-4] The isotope effect data indicate that the σ-bond to the leaving group is also considerably weakened in the transition states of the thiolate reactions, making addition intermediates unlikely. There is greater loss of the carbonyl π-bond for the reactions with thiolates than with oxyanion nucleophiles, as well as a different distribution of electron density.

Precedents for differences between the fundamental behaviors of sulfur and oxygen nucleophiles have been found in theoretical studies of hydrosulfide versus hydroxide in computational modeling of their carbonyl addition reactions to formaldehyde and formamide. A stable tetrahedral adduct was found for the hydroxide reaction, but not for that with hydrosulfide, unless attack was made on the protonated substrate.[30]



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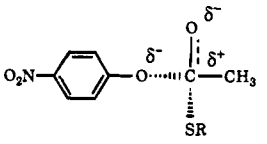

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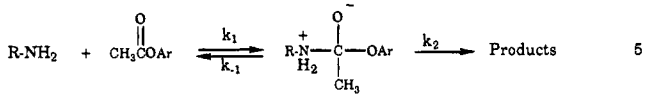
Reaction with Methoxyethylamine. The magnitudes of the leaving group isotope effects for this reaction require that the bond to the leaving group breaks in the rate-limiting step. This is consistent either with a concerted mechanism or with one involving rate-limiting breakdown of an intermediate. Such a partitioning pathway for a zwitterionic tetrahedral intermediate, which was ruled out for the anionic intermediates other nucleophiles used in this study, is plausible for the amine nucleophile because it can leave as a neutral species and therefore will be a better leaving group than suggested solely on the basis of its pK_a. This could result in a significant magnitude for k_{-1} in eq 5.



7 5

This reaction falls into the class of phenyl ester aminolysis reactions that have been proposed to proceed through rate-limiting breakdown of the zwitterionic tetrahedral intermediate **7** in eq 5, with no significant loss of a proton having occurred in the transition state.[31] The pK_a of the protons on the nitrogen atom in **7** has been estimated to be 9.6.[31] If this is accurate, then the pH of reaction (9.0) will favor the zwitterionic species **7** over the anionic species that would result from deprotonation.

In such a mechanism where an intermediate is rapidly and reversibly formed, the observed isotope effects will reflect the equilibrium isotope effects on formation of the tetrahedral intermediate and the kinetic effects on its decomposition. A unique resolution of these effects is not possible, but the observed isotope effects are consistent with the proposed mechanism where the bond to the leaving group is broken in the rate-limiting step. The magnitudes of $^{18}k_{lg}$ and ^{15}k indicate that bond cleavage to the leaving group is well advanced in the transition state. The large size of $^{18}k_{lg}$ compared with the data from the other nucleophiles may indicate a later transition state or could reflect the proximity in the transition state of the departing phenolate anion and the positively charged amide. This would stabilize more of the negative charge in the leaving group on the phenolic oxygen atom, which would offset the tendency of $^{18}k_{lg}$ to be reduced by charge delocalization effects discussed earlier. If the reaction is stepwise, a fairly late transition state for breakdown of the intermediate is also supported by $^{18}k_{carbonyl}$ and ^{D}k, both of which are considerably diminished from their expected equilibrium values for formation of a tetrahedral intermediate, presumably due to the kinetic effects on its breakdown, which will be in the opposite direction from its formation.

Conclusions

The data for the reactions of oxyanion nucleophiles with PNPA support a concerted mechanism, in agreement with the conclusions from Brønsted and theoretical studies with phenolate nucleophiles. The isotope effect data reveal no substantive difference in transition state structure between the reaction with phenolate and that with an aliphatic nucleophile of similar pK_a. The carbonyl π-bond is slightly weakened in the transition states of these reactions, indicating some tetrahedral character, but the dominant change is cleavage of the bond to the leaving group. The transition state is earlier for the reaction with hydroxide than those with oxyanion nucleophiles of lower pK_a values.

Reactions of thiolate anions with PNPA reveal a considerably different transition state structure than for oxyanion nucleophiles. These exhibit greater transition state weakening of the carbonyl π-bond, with a similar degree of bond cleavage to the leaving group as with oxyanion nucleophiles. In addition a much different charge distribution is indicated, with the maintenance of greater partial positive charge on the carbonyl carbon atom.

The data for the reaction of PNPA with methoxyethylamine do not distinguish between a concerted mechanism and one with rate-limiting breakdown of a zwitterionic tetrahedral intermediate but require that bond cleavage to the leaving group occurs in the rate-limiting step.

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