of 90 atom % ^{13}C was administered to each of three flasks containing 100 mL of growing cultures. The ^{13}C NMR spectrum which is shown in Figure 2 was derived from this experiment.

Incorporation of $^{13}CO_2$ into Cycloheximide. The general procedure followed that described for the administration of the ^{13}C-labeled acetates. At the 96-h period 1 mL of a solution containing 150 mg of $Na_2{}^{13}CO_3$ was administered to each of three growing cultures of *S. griseus*. The fermentation was terminated at 120 h and the ^{13}C-enriched cycloheximide isolated as described above.

T_1's and Effect of the Addition of 2,6-Lutidine on the ^{13}C-Carbonyl Shifts of 14. The ^{13}C spectra of cyclohexane carboxylic acid and valerolactone were obtained in D_2O–dioxane and gave ^{13}C-carbonyl shifts at 179.3 and 179.6 ppm, respectively. Addition of an excess (~3 M) of 2,6-lutidine to each of these solutions gave the following changes in carbonyl shifts: cyclohexanecarboxylic acid, ^{13}CO = 179.69 ($\Delta\delta$ = +1.39 ppm); valerolactone, ^{13}C = 179.32 ppm ($\Delta\delta$ = −0.27 ppm). The analogous experiment with the lactone-acid **16** gave the following results: ^{13}CO shifts in D_2O–dioxane: C(2) carboxylic acid 174.6 ppm and C(6) lactone 173.6 ppm. With the addition of 3 M excess of 2.6-lutidine the following changes were observed: C(2), 176.6 ppm ($\Delta\delta$ = +2.0 ppm); C(6), 171.1 ppm ($\Delta\delta$ = 0.1 ppm).

The T_1 values obtained by the inversion-recovery process with a 180–τ–90 sequence for **14** in acetone-d_6 were as follows: C(2) carboxylic acid, 13.0 s; C(6) lactone, 9.4 s.

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Catalytic Mechanisms of Acyl Transfer Reactions in Dipolar Aprotic Media. 2. Electrophilic Activation of the Carbonyl Group by Quaternary Alkylammonium and Imidazolium Functions[1]

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Abstract: Crown ether solvated potassium acetate in dipolar aprotic media readily converts *p*-nitrophenyl *o*-toluate to the corresponding acetyltoluyl mixed anhydride. The reaction proceeds at room temperature in quantitative yield. The rate of acyl transfer is 0.0118 M^{-1} s^{-1} in acetonitrile, 0.0223 M^{-1} s^{-1} in dimethyl sulfoxide, and 0.0898 M^{-1} s^{-1} in dimethylformamide, exhibiting strictly bimolecular kinetics up to the solubility limit of the desolvated nucleophile. The reaction shows absolute dependence on dipolar aprotic media; addition of hydroxylic solvents results in strong inhibition. Formation of the mixed anhydride is efficiently catalyzed by imidazolium and quaternary ammonium neighboring groups located in close proximity to the scissile carbonyl oxygen. The electrophilic catalysis results in 1000-fold rate enhancements. The acceleratory participation of the cationic neighboring groups is consistent with stabilization of the developing negative charge at the reaction center in the course of formation of the tetrahedral intermediate. The catalytic rates are one order of magnitude faster than the aminolytic cleavage of *p*-nitrophenyl acetate by benzamidine in nonprotic media. They also exceed the rate of hydrolysis of *p*-nitrophenyl acetate by hydroxide ion in water. The mechanism of the reaction is being investigated in reference to the catalytic hydrolyses by proteolytic and lipolytic enzymes which have no serine residues at the active site.

Introduction

X-ray crystallographic studies of the past several years have clearly demonstrated that the active sites of proteolytic as well as lipolytic enzymes contain hydrophobic regions.[2,3] The fact that rather low concentrations of aqueous portions are found at the catalytic centers may indicate that the medium of enzyme-catalyzed nucleophilic reactions of carboxylic acid derivatives is aprotic in nature. The mechanistic significance and possible catalytic advantage of transferring these hydrolytic reactions into nonpolar media have remained essentially unexplored.[2]

In contrast to the abundance of bioorganic model studies of hydrolytic reactions in aqueous solutions[4] and more recent work involving micellar systems,[5] relatively few attempts have been made to elucidate the mechanism of catalytic acyl transfer reactions in nonpolar or dipolar aprotic media.[6] The limited data presently available focuses almost entirely on aminolysis of esters,[2,6-9] clearly indicating that there are substantial changes both in the reactivities of the nucleophiles[9] and in the manner by which the substituents influence the reaction rates in nonprotic vs. aqueous solutions. It appears, for example, that the rate-limiting step of the reaction between *p*-nitrophenyl acetate and pyrrolidine in acetonitrile involves the collapse rather than the formation of the tetrahedral intermediate.[6a] Analogous results were obtained in imidazole and piperidine aminolyses of *p*-nitrophenyl esters in toluene and benzene.[6b,7] These conclusions are further supported by the observation that aminolytic reactions in nonprotic media are more sensitive to substituent effects on the leaving group than on the

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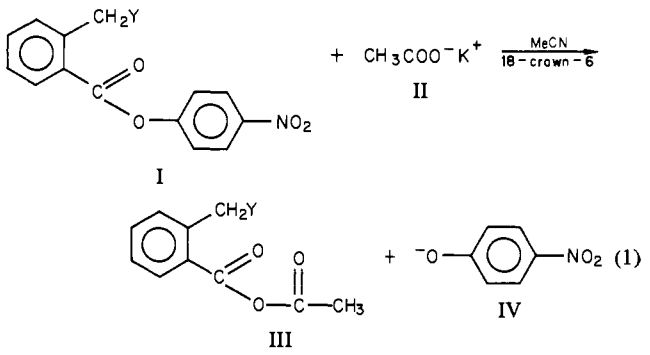
acyl moiety of the ester.[2] In addition, there is evidence that base-catalyzed deprotonation of the quaternary nitrogen in the tetrahedral intermediate substantially enhances the rate of formation of acylimidazole in the reaction between imidazole and *p*-nitrophenyl esters.[6b]

While these findings are of fundamental mechanistic significance, from the enzymological-catalytic point of view, one of the most important questions concerns the role of the medium in potentiating catalytic interactions, including the formation of reactive intermediates, that might be involved in the enzymatic breakdown of amides and esters.

In addressing this question, we have recently reported[10] that "naked" carboxylate ions, in anhydrous acetonitrile, readily cleave *p*-nitrophenyl esters to form mixed anhydrides (eq 1). As the



reaction is strongly inhibited by the addition of hydroxylic solvents, it represents a clear example of a system in which the direction of acyl transfer is *reversed* on transferring the reaction into dipolar aprotic media. In the present article, we report further mechanistic details of the reaction, with specific emphasis on neighboring-group interactions, leading to electrophilic activation of the scissile carbonyl function. These studies have been conducted to provide mechanistic insight and chemical precedents for the interactions involved in enzyme-catalyzed acyl transfer reactions with the catalytic participation of "buried" carboxylate residues. Such glutamate and aspartate functions have been found at the active site of metalloproteases[11] (i.e., carboxypeptidase, thermolysin), as well as other hydrolytic enzymes (i.e., phospholipase A_2[12]) which contain no catalytic serine residues.[13]

Experimental Section

Materials. *p*-Nitrophenyl *o*-toluate (I) was prepared from *o*-toluyl chloride (Alrich) and *p*-nitrophenol (Aldrich) in acetonitrile with triethylamine (Eastman) in quantitative yield. The product was recrystallized from ethanol: mp 108–109 °C; mass spectrum, *m/e* 257 (M^+). Anal. Calcd for $C_{14}H_{11}NO_4$: C, 65.35; H, 4.28; N, 5.45. Found: C, 65.23; H, 4:41; N, 5.53.

α-Bromo-*p*-nitrophenyl *o*-toluate was prepared by photobromination of I in rfluxing carbon tetrachloride.[14] *p*-Nitrophenyl *o*-toluate (6.4 g, 0.025 mol) was dissolved in 40 mL of boiling carbon tetrachloride, and 4.0 g (0.025 mol) of bromine in 40 mL of carbon tetrachloride was added dropwise, while the solution was irradiated using a 75-W tungsten lamp at 5-cm distance from the reaction vessel. When the color of the solution faded, the reaction mixture was cooled (dry ice-acetone) and 350 mL of petroleum ether was added. The white precipitate that separated was washed several times with petroleum ether, suction filtered, and vacuum dried over KOH pellets. The product (5.8 g, 69%) showed a single spot on thin-layer chromatography and gave a confirmatory NMR spectrum. It was used for the preparation of the active esters without further treatment.

2-[(*p*-Nitrophenoxy)carbonyl]benzyltrimethylammonium Bromide (V). α-Bromo-*p*-nitrophenyl *o*-toluate (1.7 g) was dissolved in 20 mL of dry (Linde 4Å molecular sieves) acetone, 0.5 g of anhydrous trimethylamine (Eastman) was added, and the reaction mixture was kept in a tightly sealed bottle at room temperature for 2 weeks. The white crystalline product that separated (1.5 g, 75%) was filtered and recrystallized from ethanol, mp 190–192 °C. The product gave a single spot on thin-layer chromatography and gave a confirmatory NMR spectrum. Volhard titration for bromide[15] gave an equivalent weight of 395.07. Anal. Calcd for $C_{17}H_{19}N_2O_4Br$: C, 51.65; H, 4.81; N, 7.09. Found: C, 51.45; H, 4.91; N, 6.99.

2-[(*p*-Nitrophenoxy)carbonyl]benzyltriethylammonium Bromide (VI) was prepared in the same manner as V using 1.7 g of α-bromo-*p*-nitrophenyl *o*-toluate with 0.7 g of anhydrous triethylamine (Eastman), and 1.9 g (86%) of VI was obtained. The product was recrystallized from ethanol, mp 181–183 °C. Thin-layer chromatography showed a single spot for the product, and the NMR spectrum was consistent with the structure. Volhard titration gave an equivalent weight of 437.9. Anal. Calcd for $C_{20}H_{25}N_2O_4Br$: C, 54.91; H, 5.72; N, 6.41. Found: C, 54.93; H, 5.92; N, 6.38.

2-[(*p*-Nitrophenoxy)carbonyl]benzyl-2′,3′-dimethylimidazolium Bromide (VII) was prepared in exactly the same way as the other quaternary ammonium substituted esters. α-Bromo-*p*-nitrophenyl *o*-toluate (1.7 g was reacted with 1.0 g of 2,3-dimethylimidazole (Aldrich) in dry acetone, yielding 1.9 g (87%) of VII. Recrystallization from ethanol gave an analytically pure product, mp 223–224 °C. Volhard titration gave an equivalent weight of 432.1. Anal. Calcd for $C_{19}H_{18}N_3O_4Br$: C, 52.78; H, 4.17; N, 9.72. Found: C, 52.65; H, 4.16; N, 9.48.

Potassium acetate (reagent grade, Fisher "certified" or J.T. Baker "analyzed"), tetramethylammonium acetate (Matheson, Coleman and Bell), 18-crown-6 (PCR, Inc., Gainesville, FL), and Kryptofix 222 (Matheson, Coleman and Bell) were used without further purification. Aniline (reagent grade J.T. Baker "analyzed") was freshly distilled before use.

Acetonitrile (Matheson, Coleman and Bell spectrograde and Burdick and Jackson spectrograde) was dried over activated molecular sieves (Linde 4Å, Ventron) for at least 36 h before use. Methanol (Matheson, Coleman and Bell spectrograde) was used as received. Dimethylformamide (Matheson, Coleman and Bell spectrograde) and dimethyl sulfoxide (Burdick and Jackson spectrograde) were dried and kept over activated molecular sieves (Linde 4Å).

Methods. All spectra were obtained with a Cary 14 spectrophotometer equipped with a thermostated cell compartment. The same instrument was used for the kinetic studies as well.

Kinetic measurements in all solvents were carried out under pseudo-first-order conditions with at least a tenfold excess of the crown ether solvated acetate. The concentrations of the *p*-nitrophenyl esters were in the $1-5 \times 10^{-5}$ M range. The reaction was initiated by mixing the reactants in a 3-mL Teflon-stoppered cuvette. The rates of acyl transfer were measured either by following the increase in absorbance at 430 nm (*p*-nitrophenolate production) or by monitoring the decrease in absorbance at 271 nm (disappearance of the ester). All reaction mixtures exhibited strict first-order kinetics over more than three half-lives when the carboxylate ion was present in large excess. For all the kinetics reported here, the observed pseudo-first-order rate constants exhibited a linear dependence on the concentration of the crown ether solvated potassium acetate up to the 0.1 M range. Experiments utilizing the 2,2,2 cryptate and tetramethylammonium acetate were carried out in a similar fashion. In control experiments the crown ether or the 2,2,2 cryptate had no effect; in the absence of potassium acetate, no cleavage of the *p*-nitrophenyl esters was observed. Addition of excess crown ether within the 0.1 M range had no effect on the rate constants.

Product Studies. Direct observation of the mixed anhydride (III) was accomplished by mixing equivalent amounts of 0.1 M *p*-nitrophenyl *o*-toluate and 0.1 M 18-crown-6 solvated potassium acetate in acetronitrile and transferring a sample of the product solution into a sodium chloride 1R cell. The infrared spectrum obtained exhibited a long-wavelength carbonyl absorption at 1765 cm^{-1}, absent in the spectra of the starting materials and consistent with the presence of *o*-toluyl acetate (III).[16]

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Table I. Second-Order Rate Constants for the Acyl Transfer Reaction between Acetate Ion and a Series of *p*-Nitrophenyl *o*-Toluates[a]

compd	substitution at the α position, Y	solvation of the counterion, X^+	k_2 (25 °C), mol^{-1} s^{-1} MeCN: $Z = 71.3$[b]	Me_2SO: $Z = 71.1$	DMF: $Z = 68.5$
Ia	H	K^+ 18-crown-6	0.0118	0.0223	0.0898
		K^+ 2,2,2[c]		0.0219	
Ib	$N(CH_3)_3^+$	K^+ 18-crown-6	32.74		71.97
		K^+ 2,2,2[c]	45.88	11.39	
Ic	$N(C_2H_5)_3^+$	K^+ 18-crown-6	36.89	21.88	
		K^+ 2,2,2[c]		14.39	

[a] All kinetic runs were carried out under pseudo-first-order conditions using 2–5 × 10^{-5} M *p*-nitrophenyl *o*-toluates. The concentrations of the acetate nucleophile used were in 10^2-fold or higher excess. [b] Taken from ref 17. [c] Kryptofix 222 (4,7,13,16,21,24-hexaoxy-1,10-diazabicyclo[8.8.8]hexacosane) was obtained from Matheson Coleman and Bell.

Hydrolytic side reactions by traces of water or other nucleophilic impurities could readily be ruled out by thin-layer chromatographic analysis of the product mixture, using an authentic sample of sodium *o*-toluate. The acyl transfer reaction was carried out by the stepwise addition of 1 equiv of *p*-nitrophenyl *o*-toluate to 10 mL of 0.1 M 18-crown-6 solvated potassium acetate. The resulting solution was then added to 2 mL of freshly distilled aniline. This product solution was spotted on thin-layer chromatographyic plates and run against independently prepared acetanilide, *o*-toluanilide, and sodium *o*-toluate. Absolutely no *o*-toluate was observed and only traces of acetanilide were detected. Furthermore, we found no traces of *o*-toluanilide in the product, and the major component was the ester *p*-nitrophenyl *o*-toluate.[19]

In a second, related experiment we added 1 equiv of tetramethylammonium acetate to 10 mL of 0.1 M *p*-nitrophenyl *o*-toluate. The optical spectrum of the product was determined to establish the completion of the reaction, and the solution was concentrated under nitrogen. Addition of excess water resulted in the precipitation of the regenerated *p*-nitrophenyl ester, which was readily isolated as the major product.

Results

Cleavage of *p*-Nitrophenyl *o*-Toluates (I) by Desolvated Acetate Ion (II). The second-order rate constants for the acyl transfer reaction between I and II in a series of dipolar aprotic solvents are reported in Table I. The rates have been determined either by following the disappearance of the *p*-nitrophenyl ester at 271 nm or by monitoring the formation of the *p*-nitrophenolate ion at 430 nm.[21] The reaction exhibits strictly second-order kinetics. A plot of k_{obsd} vs. [acetate] is linear and has a zero intercept (Figure 1). The first-order dependence of the rate of crown ether solvated potassium acetate is consistently maintained up to the 0.1 M concentration range, limited only by the solubility of the reagent in the solvent employed. Furthermore, the reaction proceeds to completion in the direction written (eq 1); for each mole of ester cleaved, 1 mol of *p*-nitrophenolate is generated.

The stoichiometry of the reaction was determined by comparison of the absorption of the reaction mixture to the spectrum of independently prepared potassium *p*-nitrophenoxide in the corresponding solvent. Formation of the mixed anhydride (III) was directly observed by recording the infrared spectrum of the product solution in acetonitrile. The 1765-cm^{-1} carbonyl peak absent in the spectra of both starting materials strongly supports the presence of the anhydride (see Product Studies).

The magnitude of the second-order rate constant of the acyl transfer reaction shows variation with the nature of the dipolar aprotic solvents. Comparison based on the Kosower solvent-polarity parameters (*Z* values)[22] indicates a moderate solvent effect: k_2 is increased from 0.0118 M^{-1} s^{-1} in acetonitrile (Z = 71.3) to 0.0898 M^{-1} s^{-1} in dimethylformamide (Z = 68.5). In agreement with this, the rate constant obtained in dimethyl sulfoxide (Z = 71.1) is 0.0223 M^{-1} s^{-1}, reasonably close to the corresponding value determined in acetonitrile.

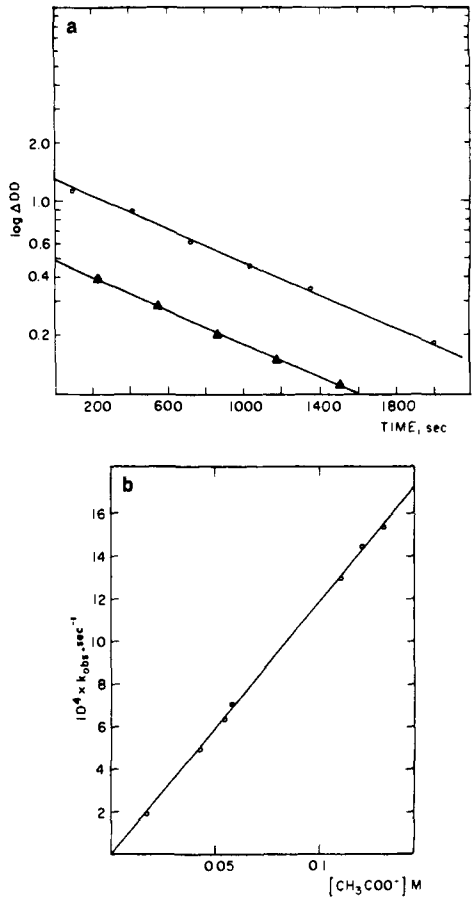

Figure 1. The kinetics of the reaction of *p*-nitrophenyl *o*-toluate and 18-crown-6 solvated potassium acetate in anhydrous acetonitrile. (a) Semilogarithmic plot of the changes in the absorption of the reaction mixture (○) formation of *p*-nitrophenoxide at 428 and (▲) disappearance of the *p*-nitrophenyl ester at 271 nm. (b) A plot of k_{obsd} vs. [acetate] for the cleavage of the *p*-nitrophenyl ester. This plot was used for the determination of the second-order rate constant (see text).

Most of our kinetic studies have been carried out using potassium acetate in conjunction with 18-crown-6 as the source of "naked" carboxylate. Replacement of the crown ether with the bicyclic complexing agent 2,2,2 cryptate, which is known to function as a more powerful potassium-sequestering agent,[23] had only marginal effect on the reaction rates. These results indicate that the nucleophilic reactivity of the carboxylate toward acyl transfer can not be further enhanced by more effective solvation of the counterion. In this context we have also examined and obtained acyl transfer with anhydrous tetramethylammonium acetate. While this reagent has been extensively used in nucleophilic S_N2 type displacement reactions for the introduction of the acetoxy group,[24] our results here demonstrate that it can function in nucleophilic additions as well, effecting facile cleavage of *p*-nitrophenyl esters in nonprotic solvents. Specifically, addition of 0.1 M tetramethylammonium acetate to 2 × 10^{-5} M *p*-nitrophenyl *o*-toluate in acetonitrile results in the formation of III with an apparent second-order rate constant of 0.0135 M^{-1} s^{-1}. Under similar conditions the corresponding second-order rate constant

(19) It has been shown in a related series of reactions involving *p*-nitrophenyl esters that the acyl transfer to acetate in acetonitrile is reversible (ref 18). The fact that a small amount of acetanilide has been produced indicates the preference of aniline toward the acetyl carbonyl of the mixed anhydride.

(20) Obviously the mechanism of the reaction between mixed anhydrides and various nucleophiles must be elucidated, with specific emphasis on delineation of the parameters which determine the reactivities and the site of attack (i.e., the scissile carbonyl) in the nuclephilic cleavage.

(21) The absorption maximum of *p*-nitrophenoxide varies with the nature of the solvent. In the presence of 18-crown-6, the λ_{max} of potassium *p*-nitropenolate is at 428 nm in acetonitrile, 433 nm in dimethylformamide, and 436 nm in dimethyl sulfoxide.

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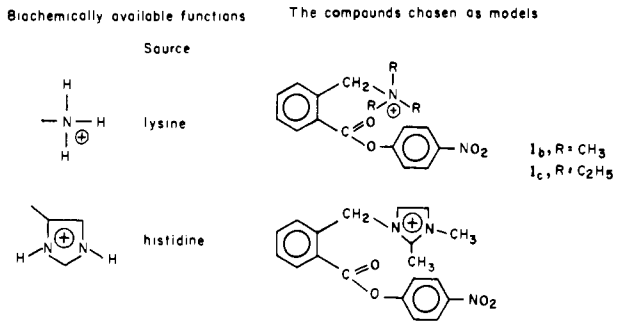
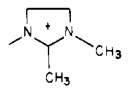


Figure 2. The model compounds chosen for studying the effect of electrophilic catalysis in the cleavage of *p*-nitrophenyl esters by acetate.

Table II. Effect of Electrophilic Catalysis on the Rates of Acyl Transfer Reaction between Substituted *p*-Nitrophenyl *o*-Toluates and Acetate[a]

compd	substitution in the α position, Y	k_2 (25 °C), mol^{-1} s^{-1} (MeCN)	k_2^{rel}
Ia	H	0.0118	1
Ib	$N^+(CH_3)_3$	32.74	2775
Ic	$N^+(C_2H_5)_3$	36.89	3127
Id		19.90	1686

[a] All kinetic runs were carried out using 18-crown-6 solvated potassium acetate ion, under pseudo-first-order conditions with excess nucleophile.

in dimethyl sulfoxide is 0.0369 M^{-1} s^{-1}, and in dimethylformamide, 0.4123 M^{-1} s^{-1} is obtained. We have not studied the kinetics of this reaction in further detail.[25]

Electrophilic Catalysis of the Acyl Transfer Reaction. The catalytic participation of positively charged neighboring groups in the reaction between *p*-nitrophenyl esters and acetate was studied using a series of functionalized model compounds, incorporating cationic residues in close proximity to the scissile carbonyl oxygen (Figure 2). The quaternary ammonium and imidazolium groups were introduced as alkyl analogues of protonated lysine and histidine residues functioning in the active site of hydrolytic enzymes.[26] The cationic esters were found to be substantially more reactive than the unsubstituted *p*-nitrophenyl toluate (Tables I and II). The rates of acyl transfer to carboxylate are enhanced 1000-fold, indicating that these neighboring groups are capable of functioning effectively as electrophilic catalysts. Moreover, the kinetic behavior of the catalytic reaction exhibits strictly first-order dependence on the nucleophile, following a similar pattern as observed for the unsubstituted ester.

In evaluating the magnitude of the rate accelerations it is important to point out that the apparent k_2^{rel} values were calculated in reference to *p*-nitrophenyl *o*-toluate without correction for the size of rather bulky alkyl-substituted quaternary ammonium groups. In addition to the steric hindrance, the model compounds possess conformational flexibility, such that the orientation and proximity between the reactive function and the catalytic moiety are considerably less than optimal. The latter is particularly apparent in dimethyl sulfoxide, where the rate enhancements are somewhat lower, probably due to more effective solvation of the cationic neighboring group by the solvent. Notwithstanding these imperfections, using alkyl analogues, rather than the corresponding protonated species, it has been possible to provide a fully quaternized nitrogen, which is stable in the presence of a strongly basic carboxylate nucleophile. This has enabled us to determine the neighboring group effects described.

Table III. Inhibition of the Acyl Transfer Reaction by Hydroxylic Solvents[a]

compd	substituent at the α position, Y	methanol added, % (v/v)	water added, % (v/v)	k_2 (25 °C), mol^{-1} s^{-1}	k_2^{rel}
Ia	H	0		0.0118	1
		0.15		0.0079	0.67
		0.3		0.0047	0.4
		1.5		0.0011	0.09
		3.0		0.0004	0.03
			0	0.0118	1
			0.8	0.0012	0.1
			1.5	0.0005	0.045
Ic	$N^+(C_2H_5)_3$	0		36.89	1
		1.0		1.84	0.05
		3.0		0.23	0.006

[a] All kinetic runs were carried out in acetonitrile, under pseudo-first-order conditions with excess nucleophile, initiating the reaction by the addition of 18-crown-6 solvated potassium acetate to the reaction mixture containing the ester and the hydroxylic solvent.

Since the catalytic reaction involves electrophilic substrate activation by a positively charged neighboring group, promoting the attack of an anionic nucleophile, the possibility of a charge–charge interaction between the reaction partners had to be considered. While the data presented in Table I cannot rule out possible contribution to the rate acceleration through localization of the carboxylate, the numbers clearly indicate similarity in the manner by which both catalytic and noncatalytic reactions respond to changes of solvent and solvation. Thus, the rather small perturbations observed in the rates on changing the nature of the potassium-chelating agent indicates that the reacting nucleophile in both cases acts as a part of a formally neutral ion pair.

Inhibition of the Reaction by Hydroxylic Solvents. The kinetic data presented in Table III indicate that the reaction between *p*-nitrophenyl esters and acetate is strongly inhibited by the addition of hydroxylic solvents. The second-order rate constant for the cleavage of *p*-nitrophenyl *o*-toluate in acetonitrile is decreased by one order of magnitude on addition of methanol, in the 1–3% concentration range. A similar tenfold decrease in the rate is observed in the presence of 0.8% water. Clearly, under these conditions the molar concentration of the protic solvent component is significantly higher (~1.0 M) than that of the acetate (0.1 M). It appears, therefore, that the inhibition results from specific solvation of the ionic nucleophile.[28]

The catalytic reaction rates involving the cleavage of the cationic esters exhibit greater sensitivity to the addition of hydroxylic solvents. In the presence of 3% methanol, the rate of acyl transfer between Ic and II is decreased 160 times. The more pronounced inhibition observed for this reaction seems to indicate that both ionic species, including the caroxylate nucleophile as well as the cationic neighboring group of the scissile ester, are solvated by

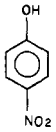
(25) We have not conducted detailed kinetic studies using tetramethylammonium acetate because of anticipated complications resulting from concentration-dependent association of the reagent in nonprotic media (see Szwarc, M. in "Ions and Ion Pairs in Organic Reactions"; Vol. I, Szwarc, M., Ed., Wiley-Interscience: New York, 1972; pp 1–26.

(26) (a) Sigman, D. S.; Mooser, G. *Annu. Rev. Biochem.* **1975**, *44*, 889. (b) Drenth, J.; Enzig, C. M.; Kalk, K. H.; Vessies, J. C. A. *Nature (London)* **1976**, *264*, 373–377.

(27) A similar type of dilemma has been encountered in conjunction with metal-ion-catalyzed nucleophilic addition reactions. The problem involves distinction between "internal" vs. "external" attack of the anionic nucleophile assisted by the complexing metal ion (for a detailed discussion, see ref 4a, pp 221–228).

(28) Specific solvation of ionic functions in dipolar aprotic media is well established; alcoholation and hydration constants of carboxylate ions, for example, have been determined (see Kolthoff, I. M.; Chantooni, M. K., Jr. *J. Am. Chem. Soc.* **1976**, *98*, 7465–7470).

Table IV. Dissociation Constants of a Series of Carboxylic Acids and Phenols in Aqueous and Dipolar Aprotic Solvents

compd	pK_a		
	H_2O	DMF[a]	Me_2SO[b]
CH_3COOH	4.75	14.2	11.9
	4.2	12.28	10.8
	7.2	12.19	10.4
	9.9	15.4	

[a] Juillard, *J. Chim. Phys. Chim. Biol.* 1970, *67*, 961. [b] Ritchie, C. D. in "Solute-Solvent Interactions"; Coetzee, J. F.; Ritchie, C. D., Eds.; Marcel Dekker: New York, 1976; Vol. II, pp 229-270.

the hydroxylic component. Based on product analyses we have found no competing side reactions that would involve direct nucleophilic addition of methanol or water.

Furthermore, we have found that in addition to hydroxylic solvents, amines have a similar effect on the acyl transfer reaction. Thus, when aniline is added it behaves as a protic solvent rather than a nucleophile; in an acetonitrile solution of III and IV, it fails to trap the mixed anhydride in the form of the corresponding anilide. It reverses the reaction instead, resulting in the formation of the *p*-nitrophenyl ester (see Product Studies).[10,19] This is in good agreement with the low nucleophilic reactivity of amines observed in nonprotic solvents as previously reported.[6]

Discussion

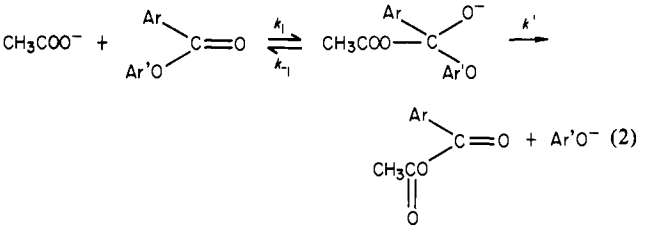
The nucleophilic cleavage of *p*-nitrophenyl *o*-toluate by desolvated acetate ion has provided the first example of a solvent-reversed acyl transfer reaction. The absolute dependence of the formation of the mixed anhydride on the dipolar aprotic media clearly demonstrates the importance of solvent participation not only in enhancing the reactivity of the anionic nucleophile, but also in altering the direction of the nucleophilic substitution reaction. These observations, together with X-ray crystallographic data, which indicate that the catalytic site of hydrolytic enzymes contains apolar regions,[2] strongly suggest that the nature of the microenvironment is an important feature of enzyme-catalyzed hydrolytic reaction mechanisms.

More specifically, the significance of the reaction here described is in providing a physical organic model for the possible mechanism of enzyme-catalyzed acyl transfer, involving the participation of a "buried" carboxylate nucleophile. Such glutamate and aspartate residues have been found at the active site of metalloproteases as well as lipolytic ester-hydrolyzing enzymes, which contain no catalytic serine residues.[11-13]

As our results indicate, the acyl transfer from *p*-nitrophenyl *o*-toluate to acetate proceeds to completion in a series of nonprotic solvents. Considering the source of the driving force for the reaction, it is pertinent to compare the changes in the relative pK_a values of the reacting nucleophiles when the system is transferred from aqueous to dipolar aprotic media. The data compiled in Table IV indicate that in aqueous solution, *p*-nitrophenolate is 2.45 pK_a units more basic than acetate. In dimethyl sulfoxide, on the other hand, the order is reversed in favor of the carboxylate, whose pK_a is now higher than that of the aromatic anion by 1.5 logarithmic units. Similarly, in dimethylformamide, acetate is 100 times more basic than *p*-nitrophenoxide. This trend, reflecting equilibrium constants, implies that the relative stabilities of the corresponding anions are reversed in nonprotic vs. aqueous solutions. The numbers can readily be understood if one considers that in the absence of hydrogen bonding to the solvent, the rather localized negative charge on the carboxylate-oxygen atoms is a great deal more destabilized than the *p*-nitrophenoxide ion, whose negative charge can be extensively delocalized by the aromatic ring. In support of this interpretation, one finds that benzoic acid in dimethylformamide is two orders of magnitude more acidic than acetic acid, while in aqueous solution the difference in the pK_a values is only 0.55.

The kinetic data clearly demonstrate that impaired solvation substantially enhances the nucleophilic reactivity of acetate. These findings are in good agreement with the well-established behavior observed for anionic nucleophiles in S_N2-type displacement reactions at saturated carbon.[8] Considering acyl transfer reactions, however, the only comparable system which has been studied in dipolar aprotic media involves the aminolytic cleavage of esters.[6,7] While formally related, the two types of nucleophilic acyl transfer reactions are quite different, both kinetically as well as thermodynamically.

In contrast to the generally accepted multiple-term kinetic scheme describing the aminolysis of *p*-nitrophenyl esters in nonprotic solvents,[6] acyl transfer to acetate from the same type of donors exhibits a strictly bimolecular pattern. Furthermore, cleavage of *p*-nitrophenyl esters by carboxylate ions occurs two orders of magnitude more rapidly than the corresponding acyl transfer reaction to amine nucleophiles. In addition, aminolyses in aprotic solvents have been shown to proceed through rate-limiting decomposition of the tetrahedral intermediate. The reaction is subject to general-base catalysis involving deprotonation of the nitrogen in the zwitterionic intermediate.[6,7] At the same time, acyl transfer to carboxylate under nonprotic conditions is efficiently promoted by electrophilic catalysis (see Table II), most probably by facilitating the development of the negative charge in the transition state, prior to the formation of the tetrahedral intermediate (eq 2). The electrostatic interaction between the carbonyl



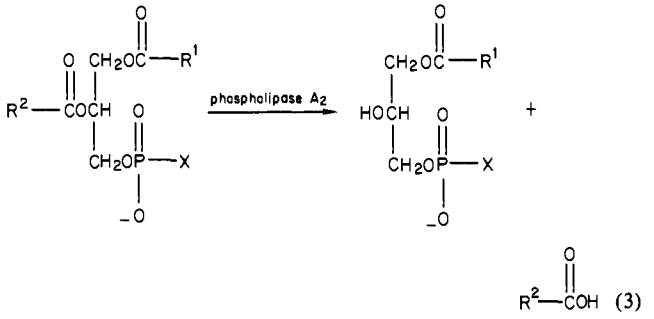
(2)

oxygen and the cationic neighboring groups in close proximity to the reactive function should lower the free energy of activation of the reaction. It is important to point out that the catalytic rate accelerations occur while both reaction partners (i.e., the acetate nucleophile and the positively charged catalytic moiety of the scissile ester) are present as part of two formally neutral ion pairs. Thus, while one cannot rule out the possibility that part of the catalytic contribution by the neighboring cationic function is providing a "localized carboxylate" at the reaction center, the observation that the rates are insensitive to the state of solvation of the potassium counterion argues against it.[27] Although a complete description of the mechanistic details, including delineation of the rate-limiting step of the reaction, has not yet been accomplished, the catalytic system here presented provides a clear example of efficient electrophilic activation of a scissile carbonyl function by a quaternary ammonium group. The rates of acyl transfer are remarkably high. Cleavage of the *p*-nitrophenyl esters (Ib–d) by acetate is 10 times faster than the aminolysis of *p*-nitrophenyl acetate by the bifunctional nucleophile benzamidine (hitherto regarded as the most efficient acyl transfer reaction in nonprotic media) and three times as rapid as the hydrolysis of the same ester by hydroxide ion in water.[9]

One of the most important distinctions between the aminolytic reaction and the carboxyl cleavage of *p*-nitrophenyl esters relates to the thermodynamics involved in the reactions considered. In contrast to the formation of the thermodynamically stable carboxylic acid derivative (i.e., amide) generated in the former reaction, acyl transfer to carboxylate produces a mixed anhydride which is less stable than the starting ester. Thus, the latter may well be considered as a reactive intermediate in the catalytic

hydrolysis of esters to carboxylic acids. Indeed, the criteria for direct nucleophilic catalysis as enunciated by Jencks[4b] are eminently fulfilled in the system here presented: (1) the desolvated carboxylate ion is more nucleophilic than the final acyl acceptor (i.e., water); (2) the intermediate mixed anhydride is more reactive than the starting ester; and (3) the intermediate is thermodynamically less stable than the final product (i.e., the carboxylic acid).

The biochemical significance of the reaction between "naked" acetate and *p*-nitrophenyl *o*-toluate is in that it provides a chemical precedent for a possible route by which active-site carboxylates might participate in enzyme-catalyzed acyl transfer reactions. Mechanistic proposals implicating "buried" residues of metalloproteases acting as direct nucleophiles have been put forward in connection with carboxypeptidase A and thermolysin.[11] It appears, however, that the system here described is more closely related to the possible mechanism of action ester-hydrolyzing lipolytic enzymes such as phospholipase A_2 (eq 3). Structural analysis



(3)

has shown that this enzyme has no catalytic serine residues. X-ray crystallographic data on pancreatic porcine phospholipase have revealed that an aspartate residue might be involved in the catalytic functioning of the enzyme.[3] This has been further supported by chemical-modification studies implicating an essential carboxylate residue in the pancreatic phospholipase.[12] Thus, formation of a catalytically competent acyl-enzyme intermediate in the form of an anhydride might be a particularly attractive proposal for the first step in the enzymatic hydrolysis of the acyl linkage at the glycerol 2 position of the phospholipid substrate. In analogy with the amide to ester interconversion involved in the mechanism of serine proteases, the ester to anhydride sequence could provide a catalytically feasible path en route to the final products, including the free fatty acid and the secondary alcohol moiety. The conformational flexibility of the protein could readily provide the suitable microenvironment necessary for the formation and subsequent decomposition of the acyl-enzyme anhydride. It is important to point out that in order to fully understand the mechanistic implications of such a mixed-anhydride intermediate in enzyme-catalyzed acyl transfer reactions, further studies of the chemistry of mixed anhydrides in dipolar aprotic media must be carried out.[20]

In conclusion, it might be noted that the reaction discussed in this work demonstrates the facility with which catalytic acyl transfer reactions can occur in nonprotic media. These findings provide additional evidence in support of the view that the transition states of the corresponding enzymatic reactions are apolar in nature.[9] Indeed, as it has been pointed our recently,[2] none of the currently accepted mechanisms for enzyme-catalyzed hydrolytic reactions involve charge separation at the rate-limiting transition states. Further studies presently underway in our laboratory are aimed at the detailed mechanistic elucidation of carboxylate-dependent acyl transfer reactions. It is hoped that these efforts will lead to a better understanding of the mechanism of this fundamentally important reaction.

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Reaction Kinetics and Equilibria of β-Elimination of Some Schiff Base Complexes

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Abstract: The kinetics of β-elimination of *O*-phosphoserine, β-chloroalanine, *S*-ethylcysteine, and β-chloro-α-aminobutyric acid was investigated by NMR in deuterium oxide media at 31.5 ± 0.5 °C in the presence of pyridoxal and in the presence and the absence of Ga(III), Al(III), and Zn(II) ions. The reaction rate constants and relevant equilibrium constants are reported for these systems. The specific rate constants for the individual molecular species in solution were determined from the observed reaction rate constants and the equilibrium constants applicable to these systems. Catalysis by pyridoxal in the absence of metal ions showed an increase in rate as pD was increased, with a rate maximum in the region of pD 8–9, and a moderate decrease at higher pH. The first-order rate constants varied with the amino acid moiety in the order β-chloroalanine > *O*-phosphoserine > β-chloro-α-aminobutyric acid > *S*-ethylcysteine. The rate constants reported for pyridoxal catalysis in the presence of metal ions show rate enhancements in the order of ten times the values of metal-free systems. Possible mechanisms of these reactions are discussed.

A series of potentiometric and spectrophotometric studies of amino acid Schiff bases of pyridoxal and pyridoxal 5′-phosphate in aqueous media have been carried out. Earlier equilibrium studies on these systems consisted of potentiometric measurements of hydrogen ion concentration supplemented by spectrophotometric data.[2,3] Because of the complex pH dependence in these proton- and metal ion-coordinated Schiff base systems, only the pH-dependent conditional rate constants have been reported.[4,5] With the introduction of computer-assisted equilibrium calculations,

(1) Abstracted in part from a dissertation submitted to the Faculty of Texas A&M University in partial fulfillment for the requirements of the degree of Doctor of Philosophy.

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