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Enhanced Rates of Acyl Transfer to a Neighboring Hydroxyl Group

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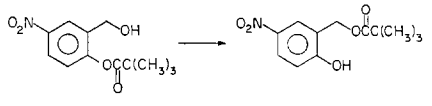
2-Hydroxymethyl-4-nitrophenyl trimethylacetate is rapidly converted, by an intramolecular pathway, to its benzyl ester counterpart in aqueous solutions of dilute buffers. Intramolecular acyl migration is favored by a factor of 10^5 over intermolecular transfer of the trimethylacetyl group to surrounding water molecules. The activation parameters of the reaction demonstrate that the rate acceleration is primarily entropic in origin. At constant pH, the apparent first-order rate constant for intramolecular acyl migration displays a linear dependence on the concentration of the basic component of the buffer. For catalysis by imidazole, a solvent deuterium isotope effect of $k_H/k_D = 2.4$ is observed, in accord with a general base-catalyzed pathway. Similarities between intramolecular and intracomplex transacylations are discussed with the conclusion that the migration of a trimethylacetyl group from the phenolic oxygen atom of a 2-hydroxymethyl-4-nitrophenol to the adjacent benzylic oxygen atom provides an accurate model for acylation of the serine hydroxyl group at the active site of α-chymotrypsin by nitrophenyl esters.

INTRODUCTION

A primary process in the reaction of α-chymotrypsin with carboxylic acid esters is the transfer of an acyl group from the ester to the hydroxyl group of a serine residue at the enzyme active site (*1*). For reaction of α-chymotrypsin with specific substrates, a mechanism involving general base catalysis of acyl transfer by an active site imidazole group is widely accepted. For reaction of α-chymotrypsin with nonspecific substrates, especially 4-nitrophenyl esters, the general base-catalyzed mechanism has been questioned. In its place, a mechanism involving nucleophilic catalysis of acyl transfer has been suggested (*2*).

Hesitancy to accept a general base-catalyzed mechanism for reaction of α-chymotrypsin with nonspecific substrates is based, in part, on the failure to observe general base catalysis of acyl transfer in appropriate model systems. Hydrolysis of 4-nitrophenyl esters, for example, is subject to nucleophilic catalysis by imidazole, not general base catalysis, as are the reactions of most carboxylic acid esters having good leaving groups (*3*).

In this paper, we report our observations concerning the rapid conversion of 2-hydroxymethyl-4-nitrophenyl trimethylacetate **1** to its benzyl ester counterpart **2**, a



1 **2**

model for reaction of α-chymotrypsin with 4-nitrophenyl esters in which general base catalysis of acyl transfer is, in fact, observed. A portion of these results has been communicated previously (*4*).

EXPERIMENTAL

Synthetic Methods

For the preparation of **1**, 5-nitrosalicylaldehyde was reduced with sodium borohydride. 2-Hydroxymethyl-4-nitrophenol, the reduction product, was then converted to **1** with trimethylacetyl chloride. Thus, a solution of 5.0 g (0.030 mole) of 5-nitrosalicylaldehyde in 50 ml of 95% ethanol was treated at room temperature with 0.56 g (0.015 mole) of sodium borohydride. After 2 hr, the solution was acidified with 30 ml of 1 *N* HCl. Solvents were then removed *in vacuo* and the residue was crystallized from ethanol to give 4.6 g (90%) of 2-hydroxymethyl-4-nitrophenol: mp 128–129°C, lit (*5*); mp 127–128°C; nmr (acetone-d_6) δ 4.83 (s, 2), 6.90–8.30 (m, 3).

One gram (0.0048 mole) of the potassium salt of 2-hydroxymethyl-4-nitrophenol (*6*) was suspended in 10 ml of anhydrous ether, cooled to 0°C, then treated dropwise with 0.54 g (0.0045 mole) of trimethylacetyl chloride. After 2 hr, the mixture was warmed to 25°C, KCl and unchanged potassium 2-hydroxymethyl-4-nitrophenolate were removed by filtration, and ether was removed *in vacuo* to give 1.1 g (96%) of **1**: nmr (acetone-d_6) δ 1.10 (s, 9), 4.70 (s, 2), 6.97–8.17 (m, 3).

A solution of 1.0 g of **1** in 10 ml of acetone containing 0.10 g of water was warmed to 30°C for 48 hr. Acetone and water were removed *in vacuo* to give 1.0 g of **2** as an amorphous solid. One crystallization from benzene gave analytically pure **2**: mp 140–141°C; nmr (acetone-d_6) δ 1.10 (s, 9), 5.21 (s, 2), 7.05–8.25 (m, 3). *Anal.* Calcd for $C_{12}H_{15}NO_5$: C, 56.91; H, 5.97; N, 5.53. Found: C, 56.73; H, 6.01; N, 5.49.

Buffers

All buffers were prepared by dissolving weighed amounts of reagent grade chemicals in doubly distilled water containing 1.0×10^{-4} *M* EDTA. At least two dilutions of each buffer were prepared while maintaining ionic strength constant and equal to the ionic strength of the parent buffer by the addition of KCl. pH values were measured at 25.1°C with a Radiometer Model 26 pH meter equipped with Radiometer electrodes. All buffers were filtered through a sintered glass disk before use in the kinetic experiments.

Imidazole–imidazole·HCl buffers were prepared by partial neutralization of once recrystallized imidazole with standardized HCl. Imidazole–imidazole·DCl buffers were prepared by partial neutralization of imidazole-1*d* with standardized DCl. Ratios of imidazole-free base concentration to total imidazole concentration were calculated from the stoichiometry of the neutralization reactions. pD values were calculated from pH meter readings (*7*).

nmr Spectra

Nuclear magnetic resonance spectra were recorded on a Varian Model T-60 spectrometer and chemical shifts were measured with reference to TMS. The spectral changes

that occur during conversion of **1** to **2** in acetone-d_6 solution containing 1.0% by weight D_2O were recorded by repetitively scanning the spectrum at measured time intervals. Spectra recorded at t_0, intermediate t, and t_∞, are reproduced in Fig. 1.

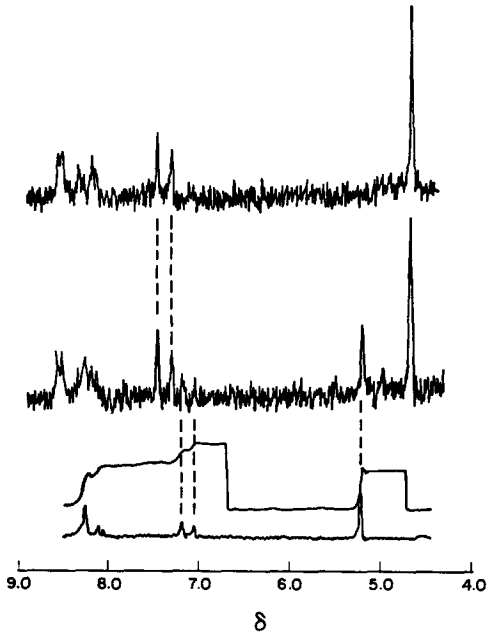


FIG. 1. From top to bottom, repetitive scans of the nmr spectrum as **1** is converted to **2**.

uv Spectra

Ultraviolet spectra were recorded on a Cary Model 14 spectrophotometer. For each measurement, 3.0 ml of a solution was placed in a glass-stoppered cell having a pathlength of 1.0 cm. Spectra of buffered solutions of **1**, **2**, and of 2-hydroxymethyl-4-nitrophenol at concentrations of 1.0×10^{-5} M or less were recorded with reference to a blank spectrum of the buffer. Whereas **1** shows little or no absorption at wavelengths greater than 300 nm, the conjugate bases of **2** and of 2-hydroxymethyl-4-nitrophenol have maxima at 405 nm (ε 18 600) and 408 nm (ε 18 700), respectively. For the ionization of **2**, an isosbestic point at 349 nm (ε 5300) is observed.

As **1** is converted to **2** in an aqueous solution buffered at pH 7.0, a smooth increase in absorption at wavelengths greater than 300 nm is observed by repetitive scanning. No evidence for the formation of an intermediate is detected. After 30 min at 25.2°C, the uv spectrum is superimposable on the spectrum of analytically pure **2** recorded under identical conditions. After 48 hr at 60°C, the product spectrum coincides with the spectrum of 2-hydroxymethyl-4-nitrophenol.

Kinetic Measurements

Conversion of **1** to **2** was monitored by recording the absorption increase at 349 nm ($\Delta\varepsilon = 5300$) as a function of time with a Cary Model 14 spectrophotometer. Thermostatted water was circulated first through the cell holder, then through the walls of the

cell compartment. Over a 1 hr period of time, the temperature of 3.0 ml of water contained in a cell positioned in the cell holder was observed to fluctuate by no more than ±0.2°C about an average temperature measured with an estimated accuracy of ±0.1°C.

For each measurement, a 1.0-cm-pathlength cell containing 3.0 ml of a buffer solution was allowed to equilibrate for 15 min at the temperature of the run. Reactions were initiated by adding from 0.010 to 0.020 ml of a 1.5×10^{-3} *M* solution of **1** in anhydrous acetonitrile to the contents of the cell. For reactions with $t_{1/2} \leqslant 60$ sec, the acetonitrile solution of **1** was placed on the flattened tip of a Teflon stirring rod. With the spectrophotometer operating, the stirring rod was immersed in the buffer solution. Mixing was accomplished by rapidly plunging the stirring rod up and down, an operation which required 3–5 sec. For slower reactions, the acetonitrile solution was pipetted directly into the cell. The cell was removed from the cell holder, stoppered, inverted five times, then reinserted into the cell holder.

In calculating rate constants, the initial 25% and final 5% of the total absorption change were discarded (*8*). Experimental values of absorption at time *t* were read from the remainder of the absorption-vs-time curve and computer-fitted to the Guggenheim equation (*9*). Computed values of absorption at infinity time always agreed with observed values to within ±2%. Nonconsecutive duplicate determinations of the rate constant usually agreed to within ±2%.

For the evaluation of activation parameters, the apparent first-order rate constant for conversion of **1** to **2** at zero buffer concentration (k_{Ψ_0}) was determined at each of five temperatures. $\Delta H^{\ddagger}/R$ was computed from the slope of a plot of $\ln(k_{\Psi_0}/T)$ against $1/T$. $\Delta S^{\ddagger}$, in turn, was calculated from the observed value of $\Delta H^{\ddagger}$ and the value of $\Delta F^{\ddagger}$ at 25.2°C.

RESULTS

Reactants and Products

Reaction of potassium 2-hydroxymethyl-4-nitrophenolate with trimethylacetyl chloride in an ethereal suspension maintained at 0°C provides **1** in quantitative yield. In moist solvents, **1** in turn is converted to **2**, which has been isolated as an analytically pure crystalline solid.

As **1** is converted to **2** in a solution of aqueous acetone, a singlet resonance in the nmr spectrum of **1** at 4.70δ decays as a new singlet resonance at 5.21δ appears (Fig. 1). This change is analogous to the change in chemical shift of the methylene hydrogens accompanying esterification of benzyl alcohol (*10*) as well as to the 4.45–5.03δ change previously reported for the conversion of the phenolic acetate of 2-hydroxymethyl-4,6-dimethylphenol to the corresponding benzylic acetate (*11*). Under the conditions employed for the nmr studies, the only reaction product is **2**, as indicated by the identity of the spectra of that product and analytically pure **2**. Resonances initially attributed to **1** cannot be detected at the completion of the reaction.

Under the conditions employed for the kinetic studies, the uv absorption spectrum of the reaction product is superimposable (with respect to wavelength of maximum absorption as well as to extinction coefficient) on the spectrum of analytically pure **2**.

On a substantially longer time scale, **2** is converted to a product whose uv spectrum coincides with the spectrum of 2-hydroxymethyl-4-nitrophenol.

We conclude from these results that, under the conditions employed for the kinetic studies, **1** is quantitatively converted to **2**. We further conclude that subsequent hydrolysis of **2** is negligible on the time scale of intramolecular transacylation.

Kinetic Measurements

Conversion of **1** to **2** in aqueous solutions of dilute buffers produces an absorption increase at 349 nm, the isosbestic point for ionization of **2**, with $\Delta\varepsilon = 5300$. The rate of change of absorption at 349 nm obeys a first-order rate law and k_ψ, the apparent first-order rate constant for conversion of **1** to **2**, has been evaluated from the absorption-vs-time curve by the method of Guggenheim (*9*). Values of k_ψ are independent of reactant concentration over a 10^{-6}–10^{-5} M range and only slightly dependent on ionic strength over a 0.05–0.50 M range. Values of k_ψ depend on pH, temperature, and buffer concentration as described below.

pH Dependence

Values of k_ψ have been determined as a function of buffer concentration at each of six pH values over a pH range of 4.95–7.60. Plots of k_ψ against total buffer concentration are linear and provide k_{ψ_0}, the apparent first-order rate constant for conversion of **1** to **2** at zero buffer concentration, as the intercept. These data are tabulated in Table 1 and a plot of the logarithm of k_{ψ_0} against pH is presented in Fig. 2.

TABLE 1

OBSERVED RATES OF TRANSACYLATION AT 25.2°C AS A FUNCTION OF pH[a]

pH	k_{ψ_0} (sec^{-1})	k_{ψ_0}/a_{OH} $\times 10^{-5}$ (M^{-1} sec^{-1})
4.95[b]	3.00×10^{-4}	3.33
5.78	2.08×10^{-3}	3.47
6.40	8.98×10^{-3}	3.59
6.81	2.30×10^{-2}	3.54
7.00	3.28×10^{-2}	3.28
7.60	1.28×10^{-1}	3.21

[a] Extrapolated to zero buffer concentration at an ionic strength of 0.20 M. All measurements were conducted in phosphate buffers unless otherwise noted.

[b] Acetate buffer.

Rate Acceleration

The ratio of the first-order rate constant for conversion of **1** to **2** to the first-order rate constant for hydrolysis of 4-nitrophenyl trimethylacetate has been employed as a measure of the efficiency of the intramolecular process. Hydrolysis of 4-nitrophenyl trimethylacetate at pH 7.60 and 25.2°C obeys a first-order rate law with $k_\psi = 1.45 \times 10^{-6}$ sec^{-1}. Under identical conditions, conversion of **1** to **2** has $k_\psi = 1.28 \times 10^{-1}$ sec^{-1}, thus providing a rate constant ratio of 9×10^4.

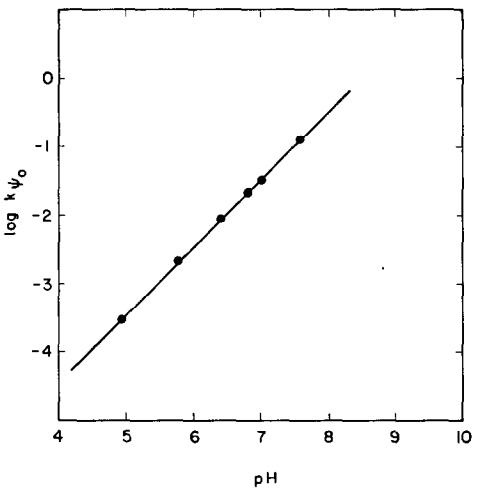


FIG. 2. The pH dependence of k_{ψ_0}.

Temperature Dependence

Values of k_ψ have been measured as a function of buffer concentration at each of five temperatures spanning a range of 13.0–35.1°C. All measurements were conducted in phosphate buffers having pH 6.81. Values of k_{ψ_0} (Table 2) were obtained by linear extrapolation to zero buffer concentration. The temperature dependence of k_{ψ_0} implies $\Delta H^\ddagger = 12.9 \pm 0.2$ kcal/mole and $\Delta S^\ddagger = -22.6 \pm 0.3$ gibbs.

TABLE 2

OBSERVED RATES OF TRANSACYLATION AT pH 6.81 AS A FUNCTION OF TEMPERATURE[a]

T (°C)	k_{ψ_0} (sec^{-1})
13.0	8.82×10^{-3}
19.7	1.51×10^{-2}
25.2	2.30×10^{-2}
30.4	3.42×10^{-2}
35.1	4.88×10^{-2}

[a] Extrapolated to zero buffer concentration at an ionic strength of 0.20 *M*. All measurements were conducted in phosphate buffers.

Catalysis by Imidazole

At fixed ratios of imidazole-free base concentration to imidazolium hydrochloride concentration, plots of k_ψ against total buffer concentration are linear. As the ratio of imidazole-free base to imidazolium hydrochloride is varied, the dependence of k_ψ on total buffer concentration increases with increasing imidazole-free base content

TABLE 3

APPARENT SECOND-ORDER RATE CONSTANTS AT 25.2°C AS A FUNCTION OF BUFFER RATIO IN H_2O AND D_2O[a]

Solvent	Buffer ratio[b]	$k_{Im-total}$[c] (M^{-1} sec^{-1})
H_2O	0.750	3.98×10^{-2}
	0.500	3.18×10^{-2}
	0.250	2.23×10^{-2}
D_2O	0.750	1.83×10^{-2}
	0.500	1.47×10^{-2}
	0.250	1.27×10^{-2}

[a] Ionic strength = 0.50 *M*.

[b] Ratio of imidazole-free base concentration to total imidazole concentration.

[c] Observed slope of a plot of k_ψ against total imidazole concentration.

TABLE 4

SECOND-ORDER RATE CONSTANTS FOR CATALYSIS BY IMIDAZOLE-FREE BASE AT 25.2°C IN H_2O AND D_2O[a]

Solvent	k_{Im} (M^{-1} sec^{-1})	$k_{Im}(H_2O)/k_{Im}(D_2O)$
H_2O	$(4.93 \pm 0.15) \times 10^{-2}$	2.4 ± 0.2
D_2O	$(2.04 \pm 0.14) \times 10^{-2}$	

[a] Ionic strength = 0.50 *M*.

(Table 3). The second-order rate constant for catalysis of acyl transfer by imidazole-free base (k_{Im}) was obtained by linear extrapolation to 100% imidazole-free base content. The measurements were repeated in D_2O to provide $k_{Im}(D_2O)$. Values of $k_{Im}(H_2O)$ and $k_{Im}(D_2O)$ are presented in Table 4 together with the value of the solvent deuterium isotope effect derived therefrom.

DISCUSSION

Intramolecular nucleophilic reactions of hydroxyl groups with carboxylic acid derivatives have received considerable attention in recent years as models for intracomplex enzymatic reactions (*4*, *12–17*). The cyclizations of 2-hydroxymethylbenzamide (*14*) and of ethyl 2-hydroxymethylbenzoate (*15*) to phthalide are particularly noteworthy as models for the reactions of amides and ethyl carboxylates with α-chymotrypsin since general base catalysis is observed. These intramolecular cyclizations as

well as intramolecular participation of a benzylic alkoxide ion in the decomposition of a carbamate ester (*16*) are favored by factors of 10^5 or more relative to their intermolecular counterparts. Such is the case for conversion of **1** to **2** in which, using the rate of hydrolysis of 4-nitrophenyl trimethylacetate as an estimate for the rate of hydrolysis of **1**, intramolecular transacylation is favored by a factor of approximately 10^5 over intermolecular transfer of the trimethylacetyl group to surrounding water molecules. In contrast to intermolecular reactions in which an unfavorable entropy term related to the loss of translational and rotational degrees of freedom must be overcome in the activation process, only a relatively small amount of entropy is lost in the activation process of an intramolecular reaction (*18*). In accord with this fact, the observed $\Delta S^{\ddagger}$ of -22.6 ± 0.3 gibbs for the conversion of **1** to **2** is more positive by 10–20 gibbs than the entropies of activation associated with intermolecular acyl transfer to uncharged reactants (*19*).

In imidazole buffers, the observed rate law indicates that, in a principal pathway for the conversion of **1** to **2**, the rate-determining activated complex contains 1 mole of **1** and 1 mole of imidazole. The solvent deuterium isotope effect, in turn, implies that an O–H bond is stretched in the transition state. On the basis of these results, we favor a mechanism in which imidazole acts as a general base catalyst for intramolecular nucleophilic attack by the adjacent benzylic hydroxyl group. We eliminate a mechanism involving nucleophilic catalysis by imidazole for the following reasons: (i) Nucleophilic catalysis by imidazole does not easily account for quantitative production of **2**; (ii) the product of intermolecular transfer of the trimethylacetyl group to imidazole is not detected in the reaction mixture; (iii) the observed second-order rate constant of $4.76 \times 10^{-2}\ M^{-1}\ \text{sec}^{-1}$ (at 25°C) for imidazole-catalyzed conversion of **1** to **2** exceeds the reported (*20*) second-order rate constant of $0.92 \times 10^{-2}\ M^{-1}\ \text{sec}^{-1}$ (at 30°C) for reaction of imidazole with 4-nitrophenyl trimethylacetate by a factor of more than 5; and (iv) nucleophilic catalysis by imidazole does not easily account for a solvent deuterium isotope effect of 2.4.

We suggest that general base catalysis of the conversion of **1** to **2** owes its existence to the availability of a potentially strong nucleophile adjacent to the ester group. In the "uncatalyzed" conversion of **1** to **2**, transfer of the trimethylacetyl group to the benzylic alcohol occurs to the exclusion of all other processes. In the catalyzed reaction, then, it is reasonable to observe that imidazole functions in such a way as to accelerate the reaction that has the lowest free energy of activation in the absence of added imidazole, namely, by acting as a general base catalyst for intramolecular nucleophilic attack of the benzylic alcohol group at the acyl carbon atom.

It can alternatively be argued that steric hindrance at the acyl carbon atom of **1** depresses a nucleophilic pathway in favor of a general base-catalyzed mechanism. This does not seem likely, however, since within a series of 4-nitrophenyl esters that includes 4-nitrophenyl trimethylacetate increasing steric hindrance at the acyl carbon atom is not sufficient to induce a changeover in mechanism from nucleophilic to general base catalysis (*20*).

Conversion of **1** to **2** differs from the reaction of α-chymotrypsin with 4-nitrophenyl esters by being an intramolecular rather than an intracomplex reaction. Otherwise, conversion of **1** to **2** is analogous to the enzymatic reaction. As in the enzymatic reaction, for example, an acyl group is transferred from a phenolic oxygen atom to the oxygen

atom of a relatively less acidic alcohol and, as in the enzymatic reaction, acyl transfer is fast. We suggest that conversion of **1** to **2** provides an accurate model for the transfer of an acyl group from a 4-nitrophenyl ester to the serine hydroxyl group at the active site of α-chymotrypsin. We then argue that the enzymatic reaction, like the conversion of **1** to **2** and like the reaction of α-chymotrypsin with specific substrates, is a general base-catalyzed process.

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