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Model Studies on the Hydrolysis of a Cyclic Ester Showing High Reactivity Toward Proteolytic Enzymes Biological Implications

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We have begun to examine the organic chemistry underlying the reversible reactions which various five-membered cyclic esters undergo with α-chymotrypsin, processes which appear to have wide biological implications. Kinetic results for the imidazole, *N*-methylimidazole and hydroxide-ion-catalyzed hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I) in normal and deuterated media have been obtained. Deuterium oxide kinetic solvent isotope effects of 4.2 and 3.5 were found for the imidazole and *N*-methylimidazole catalyzed solvolyses, respectively. Thus, it appears that these catalyses occur by a general basic rather than a nucleophilic pathway in analogy to the catalytic behavior of the imidazole residue of histidine 57 in the action of the enzyme α-chymotrypsin and in contrast to the action of imidazole as a nucleophilic catalyst in the hydrolysis of phenyl esters of carboxylic acids. An interpretation of the absence of nucleophilic catalysis in the reactions of imidazole and *N*-methylimidazole with I based on the reversibility of the formation of the corresponding sulfonyl-imidazole species is proposed.

INTRODUCTION

The five-membered cyclic esters I, II, and III[3] have been shown to react rapidly and reversibly with a serine hydroxyl (serine 195) in the active site of α-chymotrypsin to give the enzyme derivatives IV, V, and VI, respectively (*1–3*). In the reversion of the modified species IV, V, and VI to the corresponding starting cyclic esters and free enzyme the phenolic hydroxyl groups formed by the ring-opening process act as intramolecular nucleophiles which are far more effective than the external nucleophile water. For example, at pH 7 and 25.0°C the first-order rate constant for the attack of the phenolic hydroxyl of the *o*-hydroxyphenylphosphoryl group in VI on the phosphoryl phosphorus is at least 10^3 greater than the pseudo first-order rate constant for the attack of water on VI to give *o*-hydroxyphenylphosphoric acid and active α-chymotrypsin (*3*).

Our observations on the re-formation of compounds I and III from the modified chymotrypsin species are especially striking when the highly strained nature of these cyclic esters is considered. Kinetic measurements (*4, 5*) have demonstrated that cyclic esters like I and III are attacked enormously more rapidly by hydroxide ion than are their acyclic analogs. Furthermore, x-ray diffraction studies (*3, 6–9*) indicate that considerable angle strain is exhibited by five-membered cyclic sulfonates and phosphates, compounds such as I and III.

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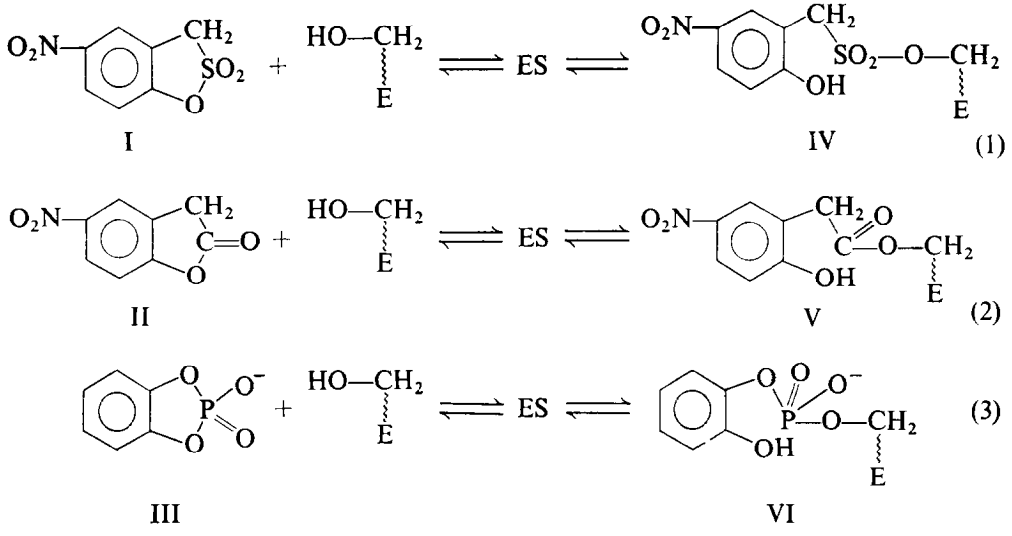
[3] In Eqs (1)–(3), ES represents the Michaelis complexes formed.



The analogy between the proximity effects of the phenolic hydroxyl groups in the decomposition of the modified enzyme species IV, V, and VI to the respective starting substances and the effect of the amino group of the newly formed amino terminal acid present in the acyltrypsin produced from the interaction of soybean trypsin inhibitor and trypsin has been discussed (*1c*, *10*). The proximity effects of the intramolecular nucleophiles present in these modified chymotrypsin and trypsin species (phenolic hydroxyl or amino groups, respectively) allow the observation under suitable circumstances of the kinetically favored product (starting cyclic ester or virgin inhibitor) rather than the thermodynamically favored product (hydrolyzed ester or hydrolyzed inhibitor). In view of the findings discussed above, the possibility that similar behavior may be involved in the action of many biologically active cyclic peptides (such as various antibiotics and hormones) and related compounds, which have the potential ability to react with enzymes either at their active sites or allosteric sites with concomitant ring opening to produce covalent acyl-enzyme species, must be considered. Under conditions where kinetic control is favored, the amino groups produced by ring-opening, which would remain covalently bound in close proximity to the acyl functions, could act as intramolecular nucleophiles, attacking the carbonyl carbons, blocking the attack of water (which would cause the destruction of the cyclic peptides), and causing the re-formation of the cyclic peptides with the release of the free enzymes (see Ref. (*11*)).[4]

To understand further the biological implications of our hypothesis and the type of enzymatic reaction seen in the formation of species IV, V, and VI from the cyclic compounds I, II, and III, our research has moved in two directions. On the one hand, we believe that it is necessary to examine in detail the chemistry of the interaction of species like cyclic AMP with appropriate enzymes. On the other, the organic chemistry underlying the enzymatic reactions which the cyclic esters undergo must be thoroughly investigated. In the present paper we wish to present the results of our initial studies on the reactions of cyclic esters with model organic catalysts.

[4] A related proposal has been made to account for the action of cyclic AMP as a second messenger in hormone action (Ref. (*3*)).

In the reversible enzymatic reactions (Eqs. (1)–(3)) of the cyclic esters with α-chymotrypsin, the imidazole moiety of histidine 57 appears to function as a general basic catalyst. We have now completed a kinetic investigation of the imidazole, *N*-methylimidazole and hydroxide-ion-catalyzed hydrolysis of the very labile sultone I in normal and deuterated aqueous media. In analogy to the behavior of the histidine group in the reaction of I with α-chymotrypsin (*1c*), the model organic catalysts imidazole and *N*-methylimidazole have been found to act as general basic rather than nucleophilic catalysts in the nonenzymatic hydrolysis of I.

EXPERIMENTAL SECTION[5]

Materials

Baker analyzed reagent grade perchloric acid was used. Fischer Scientific Co. certified sodium hydroxide solutions were employed. Matheson, Coleman, and Bell purified imidazole was recrystallized from benzene three times, mp 89.5–90.5°C. The imidazole used for the kinetic studies done in D_2O medium was treated as follows: 4 g of anhydrous, recrystallized imidazole was dissolved in 5 ml of D_2O, and the heavy water was evaporated *in vacuo* to dryness; this process was repeated at least once. Sodium perchlorate was obtained from G. F. Smith Chemical Co., Columbus, Ohio, and recrystallized three times from methanol. Deuterium oxide (99.7%) was purchased from Merck, Sharp, and Dohme of Canada Ltd. Acetonitrile (reagent grade) was distilled from P_2O_5, bp 80.5°C. *N*-methylimidazole was prepared as described in the literature (*12*) and purified by distillation from sodium. A gas chromatogram of the product showed one peak.

Imidazolium perchlorate. Slowly added to 13.6 g of imidazole in 30 ml of methanol was 17.3 ml of 70% $HClO_4$. The crystals obtained were filtered and recrystallized three times from minimum amounts of methanol, mp 309–314°C (dec) (Ref. (*13*) 302.5–307.5°C).

2-Hydroxy-5-nitro-α-toluenesulfonic acid sultone (*I*). Two and four-tenths g of 2-hydroxy-α-toluenesulfonic acid sultone (Eastman) was dissolved in 20 ml of concd H_2SO_4 and cooled in an ice bath. Concentrated HNO_3, 1.07 ml (1.53 g of 70.3% HNO_3) was added dropwise with stirring during a period of 15 min. The solution became pale yellow and was allowed to stand in an ice bath for 10 min. Ice was slowly added until no further ppt was obtained. The pale yellow ppt was filtered through a sintered glass funnel, washed with a small amount of ice cold water, and dried by suction. It was recrystallized from EtOH, mp 148.5–149.5°C (Ref. (*14*) 148°C)), yield 2.85 g (94%). *Anal.* Calcd for $C_7H_5NO_5S$: C, 39.15; H, 2.32; N, 6.25; S, 14.88. Found: C, 39.22; H, 2.27; N, 6.45; S, 14.61.[6]

Kinetic Methods

Spectrophotometric studies were made with Beckman-DU or Gilford spectrophotometers maintained at 25.0 ± 0.1°C. The reactions were followed at 400 nm. In a typical run, 50 μl of an ester solution in acetonitrile was added to a vial containing 5 ml of reaction mixture. The vial was shaken vigorously and the contents transferred to a spectrophotometer cell. $NaClO_4$ was used to keep the ionic strength constant.

[5] Infrared and nmr spectra were obtained for the compounds described and were consistent with the structures given. The instruments used were a Varian A-60 nmr spectrometer and a Perkin–Elmer Infracord. All melting points are uncorrected and were taken on a Thomas–Hoover type capillary melting-point apparatus.

[6] The microanalysis was performed by Micro-Tech Laboratories, Skokie, Illinois.

Titrimetric data were obtained for solutions at a constant pH maintained by the addition of a standard NaOH solution (Fisher certified) by means of a Radiometer Automatic titrator type TTT lb in conjunction with a type SBR2C titrigraph recorder and a pHM 25 SE pH meter equipped with a PHA 925 scale expander and a GK 2021C (NaCl) electrode. All experiments were performed at 25.0°C in a $NaClO_4$ solution under a N_2 atmosphere.

The deuterium content of the kinetic mixtures used in the D_2O experiments was always greater than 94%. In the determination of the deuteroxide ion concentrations of the reaction mixtures, the glass electrode correction formula of Fife and Bruice (*15*) and a value of $K_{D_2O} = 1.35 \times 10^{-15}$ at 25°C were employed.

RESULTS

Hydroxide catalyzed hydrolysis of I. Although the results of studies of enzyme catalyzed reactions of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone already have been reported, our experimental investigations with nonenzymatic catalysts have not been described in detail until now.

The rate of the hydrolysis of I in alkaline solution was measured at 25.0 ± 0.1°C and at ionic strength 0.8 maintained with sodium perchloride. Since the hydroxide ion concentration in a given run was maintained at a constant value using an automatic titrator, pseudo first-order kinetics were observed under these conditions. A plot of

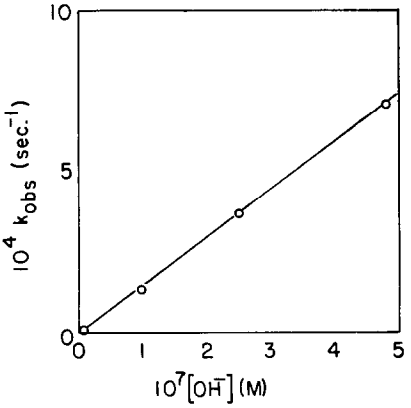


FIG. 1.

the observed pseudo first-order rate constants, k_{obsd} vs. hydroxide ion concentration is shown in Fig. 1. From the slope of the line in Fig. 1 the second order rate constant, k_{OH}, for the hydroxide ion catalyzed hydrolysis of I was determined to be 1.47×10^3 M^{-1} sec^{-1}. Since there was no appreciable intercept in the plot of Fig. 1 the contribution to the observed rates from an uncatalyzed hydrolysis reaction appears to be negligible.

To determine the effect of changes in ionic strength, the hydrolysis of I was conducted at 25.0°C in solutions containing various concentrations of sodium perchlorate. The hydroxide ion concentration was maintained at 2.51×10^{-7} M with the automatic titrator. The observed pseudo first-order rate constants are plotted in Fig. 2. It can be seen that below 0.4 M perchlorate the rate of the hydrolysis reaction increases as

the salt concentration is raised and the rate reaches a plateau in the vicinity of 0.4 *M* salt.

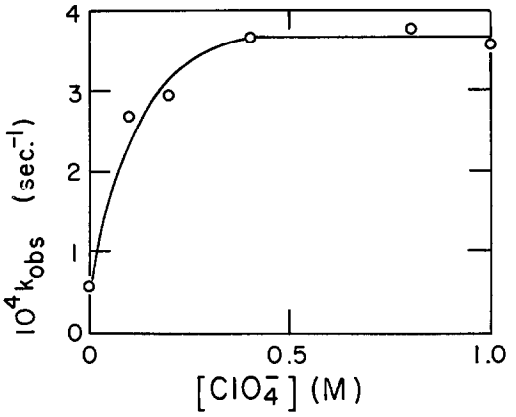


FIG. 2.

Imidazole-catalyzed hydrolysis of I. The hydrolysis of I in the presence of imidazole was studied at 25.0°C and at ionic strength 0.8 maintained with sodium perchlorate. The rate of the reaction was followed by measuring the increase in absorption at 400 nm using a Beckman DU spectrophotometer. The pseudo first-order rate constants, k_{obsd}, observed in the imidazole-catalyzed hydrolysis of I can be analyzed in terms of

$$k_{obsd} = k_{OH}(OH^-) + k_{Im}(Im) \tag{4}$$

Eq. (4). Values of the quantity $[k_{obsd} - k_{OH}(OH^-)]$ are plotted vs imidazole concentration in Fig. 3. There is obviously considerable uncertainty in the $[k_{obsd} - k_{OH}(OH^-)]$

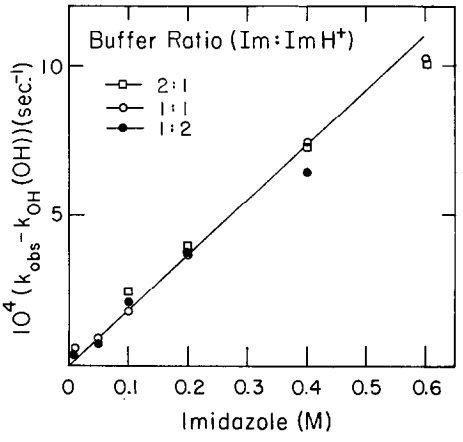


FIG. 3.

values measured at the lower imidazole concentrations since the hydroxide catalysis predominates then, and the contribution to the kinetics from catalysis by imidazole is small under these conditions. The slope of the plot in Fig. 3 gives a value of 1.7×10^{-3} M^{-1} sec^{-1} for k_{Im}.

N-*Methylimidazole-catalyzed hydrolysis of I.* The hydrolysis of I in the presence of *N*-methylimidazole was studied by the same procedure as that described above for imidazole. Pseudo first-order kinetics were observed at given *N*-methylimidazole concentrations, and the quantity $[k_{obsd} - k_{OH}(OH^-)]$ is plotted against the *N*-methylimidazole concentration in Fig. 4. The second-order rate constant for *N*-methylimidazole catalyzed hydrolysis, k_{MeIm}, was determined to be $1.6 \times 10^{-3}\ M^{-1}\ sec^{-1}$ from the slope of the line in Fig. 4. This rate constant is similar to that found for catalysis by imidazole, and it is clear that neither *N*-methylimidazole nor imidazole is a highly effective catalyst for the solvolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone. Indeed they are about six orders of magnitude less effective catalysts than hydroxide ion at comparable concentrations.

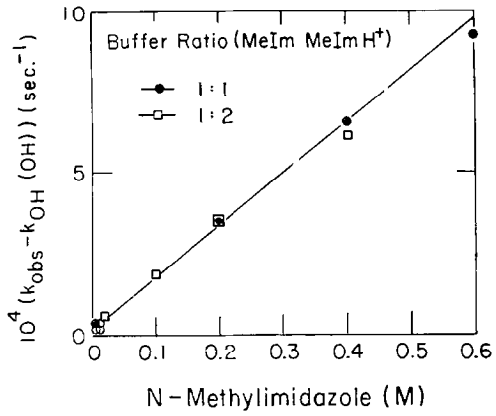


FIG. 4.

Deuteroxide, imidazole and N-*methylimidazole-catalyzed hydrolysis of I in deuterated solvent.* The determination of the second-order rate constant for the deuteroxide-catalyzed solvolysis of I was carried out at 25.0°C and ionic strength 0.8 in D_2O titrimetrically by the same method as has been described before for the hydroxide catalyzed reaction. On the basis of these measurements the k_{OH}/k_{OD} ratio for the solvolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I) at 25°C is 0.71.

The imidazole and *N*-methylimidazole-catalyzed solvolyses of I in D_2O solutions were studied by the same spectrophotometric method as was described for these reactions in unlabeled solvent. Pseudo first-order kinetics were observed at given imidazole and *N*-methylimidazole concentrations in the deuterated media. From the dependence of the quantities $[k_{obsd} - k_{OH}(OH^-)]$ on the base concentrations the second-order rate constants for the imidazole and *N*-methylimidazole-catalyzed hydrolyses in D_2O were determined to be $4.03 \times 10^{-4}\ M^{-1}\ sec^{-1}$ and $4.6 \times 10^{-4}\ M^{-1}\ sec^{-1}$, respectively. Thus, the rate-constant ratio $k_{Im}^{H_2O}/k_{Im}^{D_2O}$ for the hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I) at 25°C is 4.2, and the ratio $k_{MeIm}^{H_2O}/k_{MeIm}^{D_2O}$ is 3.5.

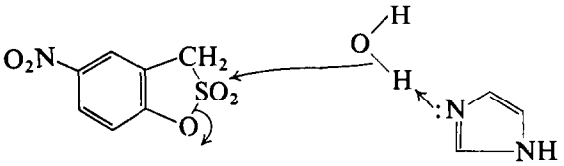
DISCUSSION

The catalysis by imidazole of the hydrolyses of carboxylic acid esters has been extensively studied. In the imidazole-catalyzed hydrolysis of *p*-nitrophenyl acetate the extent of the catalysis is a direct function of the concentration of imidazole present as

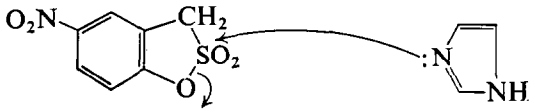
the free base (*16*). The hydrolysis of *p*-nitrophenyl acetate in the presence of imidazole has been shown to occur via nucleophilic catalysis with the intermediate formation of *N*-acetylimidazole. In general, nucleophilic catalysis has been shown to be the mechanistic pathway in the imidazole-catalyzed hydrolysis of phenyl esters of carboxylic acids (*17*). However, when acetyl esters containing very poor leaving groups were examined, a shift from nucleophilic catalysis of hydrolysis by imidazole to general base catalysis was found (*18*). As has been discussed, the change from a nucleophilic to a general base-catalyzed mechanism in the reactions of esters with imidazole can be rationalized in terms of the formation of a tetrahedral addition intermediate, although the postulation of an asymmetrical energy barrier along the reaction coordinate with no formation of an additional intermediate also can be used to explain the results (*18*, *19*).

Nucleophilic and general base catalysis of the hydrolysis of carboxylic esters by imidazole are kinetically indistinguishable processes (*17*). However, either the magnitude of the deuterium kinetic isotope effect or the detection of an acylimidazole in the case of nucleophilic catalysis can be used to distinguish between the two types of processes.

On the basis of our observations of ratios of 4.2 for $k_{Im}^{H_2O}/k_{Im}^{D_2O}$ and 3.5 for $k_{MeIm}^{H_2O}/k_{MeIm}^{D_2O}$ in the hydrolysis of the nitro-substituted sultone (I) it appears that imidazole and *N*-methylimidazole function as general basic rather than nucleophilic catalysts in this reaction. The hydrolysis of I catalyzed by imidazole can be pictured to proceed as shown in Eq. (5) below.[7]

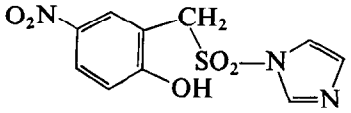


(5)



(6)

It is interesting to ask why the imidazole and *N*-methylimidazole-catalyzed reactions of I do not proceed by nucleophilic attack of the imidazole species on the sulfur atom as illustrated in Eq. (6), followed by hydrolysis of the sulfonylimidazoles formed. The excellence of the leaving group formed when the sultone ring in I is broken might



VII

[7] The reaction as illustrated is written as a concerted process, but it is certainly possible that a pentacoordinate intermediate is formed by the imidazole catalyzed attack of water, and then this intermediate decomposes with expulsion of the nitrophenoxide group to give the product, 2-hydroxy-5-nitro-α-toluenesulfonic acid. The question whether pentacoordinate intermediates occur in the solvolysis of cyclic sulfonate esters has been discussed recently (*20*).

lead one to expect on the basis of our knowledge of the imidazole catalysis of carboxylic ester solvolyses that nucleophilic catalysis of the solvolysis of I by the imidazoles as pictured in Eq. (6) rather than general base catalysis as shown in Eq. (5) should occur. However, it is possible that nucleophilic attack by imidazole to give the sulfonyl imidazole (VII) may indeed occur, but we may not detect this attack. The reason why the formation of VII may not be detected is that VII may recyclize to form the starting sultone (I) much faster than it hydrolyzes to give the product sulfonic acid. In that event, 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone could be attacked by imidazole and *N*-methylimidazole by both the routes shown in Eqs. (5) and (6), but only the former pathway would contribute significantly to the observed hydrolysis of the cyclic sulfonate (*21*).[8]

ACKNOWLEDGMENT

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[8] The preparation of VII and a study of its solvolytic reactivity obviously would be desirable. However, we have not yet succeeded in obtaining this compound although we have attempted its synthesis by several routes.