Imidazole Catalysis of Hydroxylamine Reactions with Tyrosine Esters*

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Since previous studies have suggested the possibility that general base catalysis is of importance in chymotrypsin-catalyzed reactions, it was of interest to investigate the possible involvement of this type of catalysis in chemical reactions of specific chymotrypsin substrates. The neutral hydroxylamine reactions of *N*-acetyltyrosine ethyl ester and tyrosine ethyl ester have been shown to be catalyzed by imidazole, and these catalyses are greater at *p*H 7.8 than at 7.1. The observation that the imidazole catalyses of these reactions were decreased in deuterium oxide solutions suggests the involvement of a proton transfer mechanism and the functioning of imidazole as a general base catalyst. A specific base-catalyzed hydroxylamine reaction was studied at higher *p*H values and shown not to contribute significantly to the imidazole-catalyzed reactions carried out in the neutral region. There were no apparent differences in the products obtained in the alkaline and imidazole catalyzed hydroxylamine reactions of tyrosine esters studied.

Various reactions have been studied as model systems of chymotrypsin-catalyzed reactions with respect to the roles of serine and histidine in the catalytic processes of the enzyme (Bender, 1960). The extensively studied imidazole catalysis of the hydrolysis of *p*-nitrophenyl acetate suggests the possibility of a nucleophilic catalytic role for the imidazole group of a histidine residue in chymotrypsin reactions. Imidazole catalysis of acyl transfer reactions can also occur through classical general base catalysis (Jencks and Carriuolo, 1959), and it has been suggested that general base catalysis can be another possibility for the role of imidazole in enzyme reactions (Cunningham, 1957; Dixon and Neurath, 1957; Bernhard and Gutfreund, 1958; Jencks and Carriuolo, 1959; Spencer and Sturtevant, 1959; Anderson *et al.*, 1961). The kinetic constants for the chymotrypsin-catalyzed reactions of *p*-nitrophenyl acetate and *N*-acetyltyrosine ethyl ester (Spencer and Sturtevant, 1959) demonstrate the greater ease with which the enzyme handles the more natural tyrosine substrate. It was of interest to investigate the possibility of imidazole catalysis in chemical reactions of tyrosine esters with emphasis upon the type of catalysis of importance in these reactions.

The reaction chosen for study was the neutral hydroxylamine reaction with an ester to form the corresponding hydroxamic acid, which has been reported to proceed readily with amino acid esters such as glycine ethyl ester (Bergmann and Wurzel, 1953) and leucine methyl ester (Raake, 1958). The present report describes the imidazole catalyses observed in neutral reactions of hydroxylamine with *N*-acetyltyrosine ethyl ester and tyrosine ethyl ester. A preliminary report of portions of this work has been published (Anderson, 1962).

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EXPERIMENTAL

Materials.—Tyrosine ethyl ester hydrochloride, obtained from Nutritional Biochemicals Co., was twice recrystallized from acetonitrile and dried under vacuum. *N*-Acetyltyrosine ethyl ester, chromatographically pure, was obtained from Mann Research Laboratories and used without further purification. Owing to the limited water solubility of *N*-acetyltyrosine ethyl ester a 0.033 M stock solution was prepared in 20% (v/v) ethanol. Piperidine was obtained from Matheson, Coleman and Bell, Inc. Imidazole and hydroxylamine hydrochloride were obtained from Eastman Kodak Co. Deuterium oxide (99.8%) prepared by the Atomic Energy Commission was obtained from Bio-Rad Laboratories.

Imidazole buffers were prepared by adjusting imidazole solutions to the proper *p*H with HCl. Hydroxylamine solutions at *p*H 7.1 and 7.8 were prepared by neutralizing hydroxylamine hydrochloride with sodium hydroxide immediately before use. An alkaline hydroxylamine solution was made by adding one volume of 4 N hydroxylamine hydrochloride to two volumes of 3.5 N sodium hydroxide immediately before use. Water and deuterium oxide were glass-distilled prior to use.

Methods.—The reactions of the two esters, tyrosine ethyl ester and *N*-acetyltyrosine ethyl ester, with hydroxylamine near neutrality were followed kinetically by the formation of the respective hydroxamic acids, which were measured quantitatively by the Hestrin ferric chloride method (Hestrin, 1949). These reactions were carried out in reaction mixtures containing 10 μmoles of ester, 0.3 M hydroxylamine, various

concentrations of imidazole buffers, and distilled water to make the final volume of the solution 1.0 ml. The ionic strength was maintained constant at 1.2 by the addition of KCl. Hydroxylamine preadjusted to *p*H 7.1 or 7.8 was the last reactant to be added to the reaction mixture, and prior to its addition the other reactants had been adjusted to the proper *p*H and had been brought to temperature equilibrium in a 37° constant temperature water bath. One tenth ml aliquots were removed at timed intervals and added directly to 1.0 ml ferric chloride (10% in 0.1 N HCl), and the resulting ferric-hydroxamate complex was measured at 540 mμ against an appropriate reagent blank. These reagent blanks contained the same components as the test solution with the exception of the ester, which was replaced by distilled water. The fact that incubation with concentrated alkaline hydroxylamine at the end of these reactions gave no further increase in hydroxamic acid indicated total splitting of the ester linkage. The hydroxide ion–catalyzed reaction of hydroxylamine with *N*-acetyltyrosine ethyl ester was measured in the same manner.

Kinetic studies of the alkaline hydroxylamine reaction with *N*-acetyltyrosine ethyl ester were carried out in reaction mixtures containing 60 μmoles of ester, 0.6 M hydroxylamine, 0.2 M piperidine buffers in a total volume of 6.0 ml. The reagents and reaction mixtures were prepared with glass-distilled CO_2-free water, and KCl was used to maintain the ionic strength constant at 1.2.

The alkaline hydrolysis of *N*-acetyltyrosine ethyl ester was followed both spectrophotometrically by the decrease in absorption at 254 mμ (Schwert and Takenaka, 1955) and by the decrease in alkaline hydroxylamine reactive material (Hestrin, 1949). Glass-distilled CO_2-free water was used to prepare the triethanolamine and piperidine buffers used in these studies. The reaction mixtures contained 1.65 μmoles of ester and 0.05 M buffer in a total of 5.0 ml, and KCl was added to maintain the ionic strength constant at 0.30.

The imidazole-catalyzed hydroxylamine reactions studied in deuterium oxide were carried out with the reagents described above which had been preequilibrated twice with deuterium oxide and redissolved in deuterium oxide. Distilled deuterium oxide was used in place of water in the reaction mixtures. DCl used to neutralize imidazole in these studies was prepared from deuterium oxide and phosphorus pentachloride. NaOD in deuterium oxide was used to adjust the *p*H of the hydroxylamine solutions.

Ascending paper chromatography was carried out on Whatman No. 3MM paper with two solvent systems: (1) 95% ethanol–water, 80:20 (Moldave *et al.*, 1959) and (2) 95% ethanol–0.1 N acetic acid, 1:1. The chromatograms were run for 16 hours at 25°. Spots were located by both UV absorption and by spraying with a 1:1 mixture of 10% ferric chloride in 0.5 N HCl and 95% ethanol.

Spectrophotometric measurements were carried out on a Zeiss PMQ II spectrophotometer equipped with a thermostated cell compartment. Measurements of *p*H were made with a Radiometer *p*H meter, type PHM4b with a G-200-B glass electrode. Kinetic experiments were carried out with one reactant in great excess so that pseudo-first-order kinetics was followed. Rate constants were obtained by plotting the extent of reaction, $X_\infty - X_t$, against time on semilogarithmic graph paper and by calculating the first order rate constants from the equation, $k_1 = 0.693/t_{1/2}$.

Results and Discussion

Preliminary studies on the hydrolyses of *N*-acetyltyrosine ethyl ester and tyrosine ethyl ester at *p*H 7.0 indicated that these reactions were extremely slow at 25° and 37° and the effects of imidazole on these reactions under these conditions were difficult to evaluate. The neutral hydroxylamine reactions with the tyrosine esters were readily measurable at 37° and were chosen as a means of studying imidazole catalysis in reactions of these compounds. The reaction of hydroxylamine with *N*-acetyltyrosine ethyl ester was studied at 37° in six different concentrations of imidazole buffers at *p*H 7.1 and 7.8. The reactions were carried out in the presence of an excess of hydroxylamine so that pseudo-first-order kinetics was observed and KCl was used to maintain the ionic strength constant at 1.2. The reaction is catalyzed by imidazole as shown in Figure 1, and the catalysis is greater at *p*H 7.8 than at 7.1. The curves for imidazole catalysis, when extrapolated to zero imidazole concentration, intercept at the same point, indicating no effect of *p*H on the uncatalyzed hydroxylamine reaction in the *p*H region being studied. To verify this point the hydroxylamine reaction with *N*-acetyltyrosine ethyl ester was studied under alkaline conditions to determine the *p*H range in which a hydroxide ion–catalyzed reaction could be detected. Such a specific base-catalyzed hydroxylamine reaction was observed at 25° in the *p*H range of 11–12 (Fig. 2). The experimental points here fit well with the theoretical line drawn with a slope of 1.0. The extrapolation of this line to *p*H 7.1 would indicate that a contribution from the specific base-catalyzed hydroxylamine reaction to the rate constants observed for the hydroxylamine reactions in the imidazole buffers would be insignificant. It is unlikely that the differences in the temperatures of these two reactions would have an effect great enough to invalidate this argument. The specific base-catalyzed hydrolysis of *N*-acetyltyrosine ethyl ester has been included in Figure 2 for comparison with the alkaline hydroxylamine reaction. The alkaline hydrolysis of *N*-acetyltyrosine ethyl ester was studied in both piperidine and triethanolamine buffers, and no significant differences in rate constants were

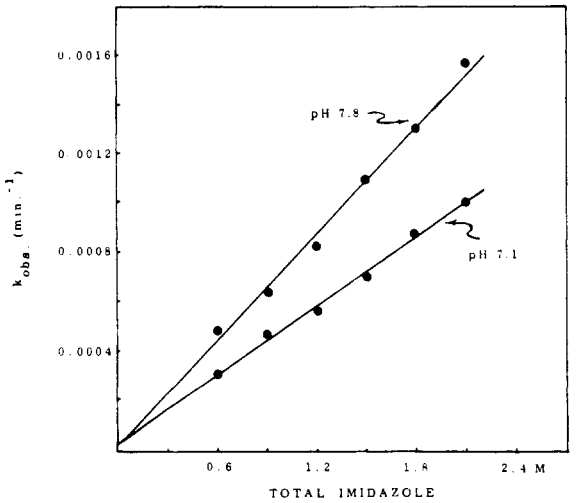

FIG. 1.—Imidazole catalysis of the hydroxylamine reaction with *N*-acetyltyrosine ethyl ester at *p*H 7.8 and 7.1, 37°, and ionic strength 1.2.

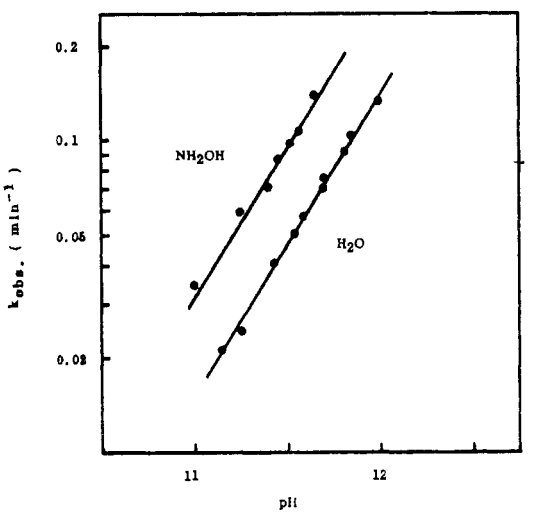

FIG. 2.—Logarithmic plots of the rates of reaction of hydroxylamine and water with *N*-acetyltyrosine ethyl ester against *p*H. The hydroxylamine reactions were carried out at 25° in 0.2 M piperidine buffers and ionic strength 1.2. The hydrolyses were carried out at 25° in 0.05 M piperidine buffers and ionic strength 0.3.

observed in these buffer systems. Varying the concentrations of these buffers from 0.08 M to 0.28 M at constant ionic strength had no effect on the rate of alkaline hydrolysis. It was also observed that the alkaline hydrolysis could be measured either spectrophotometrically by the decrease in absorption at 254 mμ or by the decrease in the alkaline hydroxylamine reactive ester without any significant differences in the rate constants obtained. The third-order rate constants for the hydroxide ion–catalyzed hydroxylamine and hydrolysis reactions of *N*-acetyltyrosine ethyl ester are 51 liter moles^{-2} min.$^{-1}$ and 15 liter^2 moles^{-2} min.$^{-1}$ respectively.

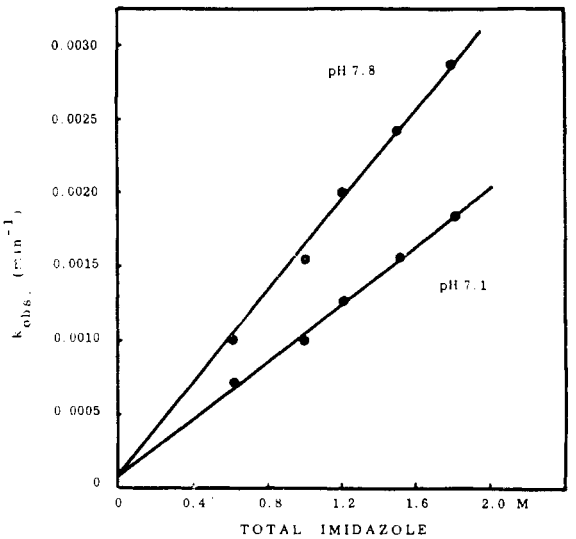

FIG. 3.—Imidazole catalysis of the hydroxylamine reaction with tyrosine ethyl ester at *p*H 7.8 and 7.1, 37°, and ionic strength 1.2.

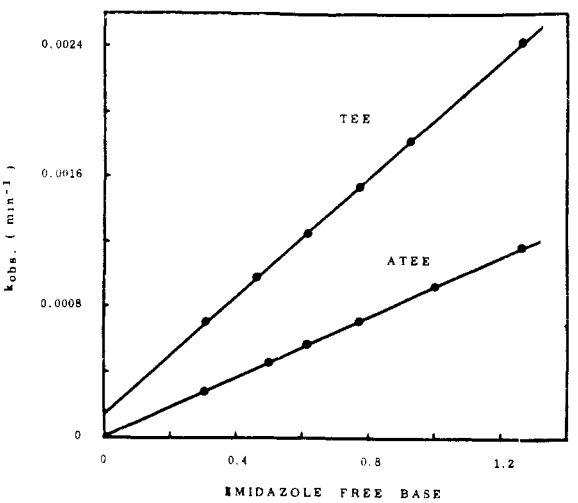

FIG. 4.—The dependence of the rates of the hydroxylamine reactions of *N*-acetyltyrosine ethyl ester and tyrosine ethyl ester on the concentration of the free base form of imidazole.

The hydroxylamine reaction of tyrosine ethyl ester was studied under identical conditions to those used with *N*-acetyltyrosine ethyl ester. Imidazole catalysis was observed in these reactions (Fig. 3), and again the catalysis by imidazole was greater at *p*H 7.8 than at *p*H 7.1. The *p*H effects observed in the imidazole catalyses of the hydroxylamine reactions of the two tyrosine esters suggest the catalytic species of imidazole in these cases to be the free base form. The observed rate constants obtained in the hydroxylamine reactions with the two tyrosine esters show a linear relationship with the concentration of imidazole free base (Fig. 4).

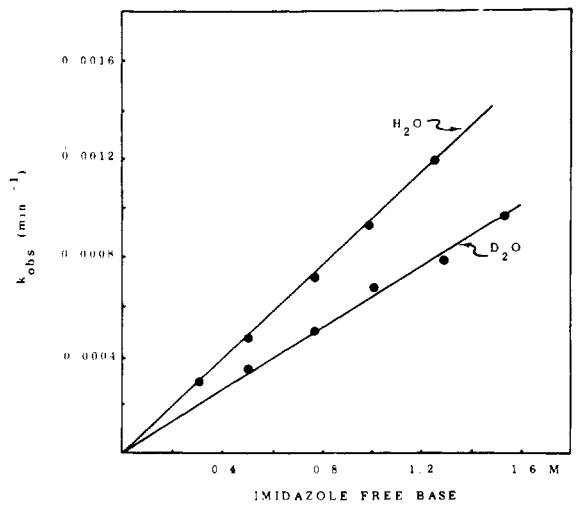

FIG. 5.—Imidazole catalysis of the hydroxylamine reactions of *N*-acetyltyrosine ethyl ester in water and deuterium oxide at 37° and ionic strength 1.2.

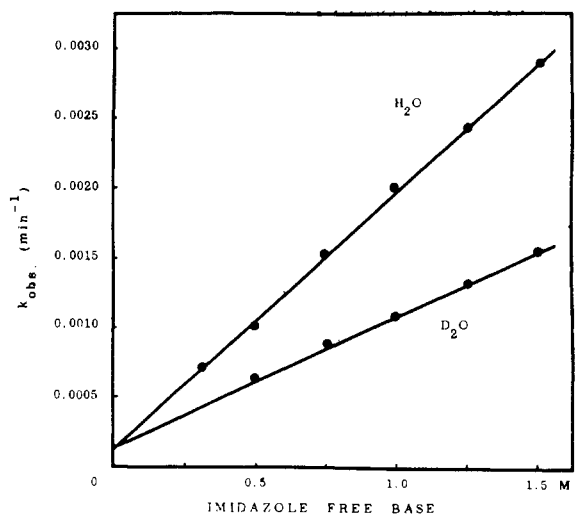

FIG. 6.—Imidazole catalysis of the hydroxylamine reactions of tyrosine ethyl ester in water and deuterium oxide at 37° and ionic strength 1.2.

The imidazole catalysis of the hydroxylamine reactions of both *N*-acetyltyrosine ethyl ester and tyrosine ethyl ester were studied in deuterium oxide under conditions identical to those of the same reactions in water. The total concentrations of imidazole in deuterium oxide solutions were obtained by complete titration of samples of these solutions and the reactions with the tyrosine esters were carried out with various concentrations of imidazole buffers neutralized 84% to the free base form. The imidazole catalysis of the hydroxylamine reaction with *N*-acetyltyrosine ethyl ester under these conditions was decreased in deuterium oxide solutions (Fig. 5). The imidazole catalysis of the hydroxylamine reaction with tyrosine ethyl ester was likewise decreased in deuterium oxide solutions (Fig. 6). The catalytic constants for imidazole in the reactions of the two tyrosine esters obtained in water and in deuterium oxide are listed in Table I. The imidazole catalysis of the *N*-acetyltyrosine ethyl ester reaction is 1.58 times greater in water than in deuterium oxide. In the case of the tyrosine ethyl ester reaction the imidazole catalysis is 1.91 times greater in water than in deuterium oxide.

Both the alkaline hydroxylamine and the imidazole catalyzed hydroxylamine reactions of tyrosine ethyl ester and *N*-acetyltyrosine ethyl ester were studied with respect to the products formed in each case. These reactions were all carried out under conditions used for the kinetic studies and were allowed to proceed essentially to completion. At the end of each reaction the reaction mixtures were adjusted with HCl to *p*H 4.6 and fractionated on Dowex-50 columns to remove the excessive amounts of nitrogen bases present. The products of these reactions were eluted from the Dowex-50 columns with water with the UV absorption of the tyrosyl moiety used as a means of localization, and the fractions containing tyrosine derivatives were immediately lyophilized. The products obtained in each reaction were compared chromatographically with two solvent systems, 80:20 95% ethanol–water (Moldave *et al.*, 1959) and 50:50 95% ethanol–0.1 N acetic acid. In both the alkaline and the imidazole catalyzed hydroxylamine reactions with tyrosine ethyl ester only one product was observed. This compound has an RF value of 0.58 in the ethanol–water system and 0.76 in the ethanol–acetic acid system; this was identical to the RF values obtained in these systems with a known sample of tyrosyl hydroxamic acid. Both hydroxylamine reactions of *N*-acetyltyrosine ethyl ester produced one major product which had an RF value of 0.48 in the ethanol-water system and 0.73 in the ethanol-acetic acid system. These RF values differed from those observed in the two solvent systems for tyrosyl hydroxamic acid (0.58 and 0.76 respectively) and acetohydroxamic acid (0.67 and 0.79 respectively), included for comparison in these studies. A second much smaller hydroxamic acid spot was observed on the chromatograms of the *N*-acetyltyrosine ethyl ester–hydroxylamine reaction mixtures. This material as yet unidentified represents only a small

TABLE I

DEUTERIUM ISOTOPE EFFECT ON IMIDAZOLE CATALYSIS OF HYDROXYLAMINE REACTIONS WITH TYROSINE ESTERS AT 37°

Ethyl Ester	$(k_{Im})^a_{H_2O}$ (M^{-1} min.$^{-1}$)	$(k_{Im})^a_{D_2O}$ (M^{-1} min.$^{-1}$)	$(k_{Im})_{H_2O}/(k_{Im})_{D_2O}$
N-Acetyltyrosine	9.60×10^{-4}	6.06×10^{-4}	1.58
Tyrosine	1.71×10^{-3}	8.98×10^{-4}	1.91

[a] $k_{Im} = \dfrac{k_{obs.} - k_0}{(\text{Imidazole})_{\text{free base}}}$ where $k_0 = k_{obs.}$ extrapolated to zero imidazole concentration.

percentage of the total hydroxamic acid present and has an R_F value greater than that of any of the hydroxamic acids studied in both solvent systems. The data obtained in the chromatographic studies are consistent with the proposal that the products formed in the imidazole-catalyzed hydroxylamine reactions with the two tyrosine esters do not differ from those obtained in the corresponding alkaline hydroxylamine reactions.

The imidazole catalysis observed in the reactions of hydroxylamine with the tyrosine esters is of interest because it represents another example of catalysis of acyl transfer reactions of "low energy" esters and because it is decreased in the presence of deuterium oxide. The fact that the imidazole catalysis of the tyrosine ester reactions with hydroxylamine was decreased in deuterium oxide solution strongly suggests that a proton transfer takes place in the activated complexes of these catalyzed reactions (Wiberg, 1955), and would indicate the functioning of imidazole here as a general base catalyst. Many other general base-catalyzed reactions such as the imidazole-catalyzed hydrolysis and acyl transfer reactions of acetylimidazole (Jencks and Carriuolo, 1959) and *N,O*-diacetylserinamide (Anderson *et al.*, 1961) as well as the imidazole-catalyzed hydrolyses of ethyl chloroacetate, ethyl dichloroacetate and ethyl difluoroacetate (Jencks and Carriuolo, 1961) are likewise decreased in deuterium oxide, as would be expected for the proton transfer type of mechanism proposed for these reactions. Similar deuterium isotope effects have been observed in other general base-catalyzed hydroxylamine reactions (Anderson *et al.*, 1961; Bruice and Bruno, 1961). The nucleophilic catalysis by imidazole of the hydrolysis of *p*-nitrophenyl acetate and phenyl acetate is not decreased in deuterium oxide solutions (Anderson *et al.*, 1961; Bender *et al.*, 1962).

Bender *et al.* (1961) have shown that deuterium oxide can cause a large decrease in the rate constants of both the acylation and deacylation steps of chymotrypsin-catalyzed reactions. The k_H/k_D for the over-all catalytic step involving a specific substrate, *N*-acetyl-L-tryptophan methyl ester, was between 2.2 and 3.1 and was interpreted as resulting from a slow proton transfer in the enzyme reaction. In this respect it is of interest that the data obtained in the chemical catalyses by imidazole of the hydroxylamine reactions with *N*-acetyltyrosine ethyl ester and tyrosine ethyl ester are consistent with a general base mechanism.

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