Oxazolinone Intermediates in the Hydrolysis of Activated *N*-Acylamino Acid Esters. The Relevance of Oxazolinones to the Mechanism of Action of Serine Proteinases*

John de Jersey,† Peter Willadsen, and Burt Zerner

ABSTRACT: The alkaline hydrolysis of *p*-nitrophenyl hippurate occurs in two steps. In the first step, which is subject to general base catalysis, *p*-nitrophenol is released and 2-phenyloxazolin-5-one is formed. Subsequent hydrolysis of this oxazolinone yields hippuric acid. The kinetics of hydrolysis of 16 esters of *N*-acylamino acids have been investigated to determine the generality of the oxazolinone mechanism. *p*-Nitrophenyl esters of formyl-, acetyl-, and cinnamoylglycine yield oxazolinone intermediates, whereas benzyloxycarbonylglycine *p*-nitrophenyl ester does not. Oxazolinone intermediates are formed from a number of activated esters of hippuric acid but not from stable esters such as methyl hippurate. The deacylation of hippuryl-chymotrypsin has been investigated under conditions where 2-phenyloxazolin-5-one could be observed if it were formed. Since no formation of oxazolinone is observed, hippuryl-chymotrypsin behaves as a *stable*, rather than as an activated, ester of an *N*-acylamino acid. The reaction of *p*-nitrophenyl hippurate with imidazole in aqueous solution yields hippurylimidazole rather than 2-phenyloxazolin-5-one.

The present understanding of the acyl-enzyme mechanism for proteolytic enzymes has been made possible by the extensive use of both stable and activated esters of *N*-acylamino acids. *p*-Nitrophenyl esters have been of special importance because of the desirable spectral properties of *p*-nitrophenol. The mechanism of hydrolysis of simple esters involves nucleophilic attack at the carbonyl carbon (Bender, 1960). However, preliminary observations on the hydrolysis of PNPH[1] revealed a more complicated mechanism involving the formation and subsequent hydrolysis of PO (de Jersey *et al.*, 1966a). In the present work, further support has been gained for the oxazolinone mechanism of hydrolysis of PNPH, and a series of *N*-acylamino acid esters has been examined in an attempt to determine the generality of the mechanism.

The acyl-enzyme intermediate in α-chymotrypsin-catalyzed hydrolyses appears to be a serine ester. The deacylation of hippuryl-chymotrypsin was therefore studied as an example of the hydrolysis of an acylamino acid ester. Because of the important role of histidine in catalysis by α-chymotrypsin, the effect of imidazole on the hydrolysis of acylamino acid esters has also been determined.

Experimental Section

Materials. Nitrophenyl esters, with one exception, were prepared by the reaction of equimolar amounts of acylamino acid, nitrophenol, and dicyclohexylcarbodiimide at room temperature in ethyl acetate. *N*-Benzoylaminoisobutyric *p*-nitrophenyl ester was prepared by the reaction of equimolar amounts of 4,4-dimethyl-2-phenyloxazolin-5-one and *p*-nitrophenol in acetonitrile, in the presence of a catalytic amount of triethylamine. Nitrophenyl esters were purified by recrystallization from chloroform–hexane or from carbon tetrachloride. Characteristics of the nitrophenyl esters prepared are given in Table I. Phenyl hippurate, prepared by the method of Weiss (1893) and recrystallized from ethanol, had mp 104° and lit. (Lowe and Williams, 1965) mp 104°. Benzyl hippurate was prepared from benzyl chloride and sodium hippurate by heating at 80–90° in the presence of a catalytic amount of triethylamine: mp 86° (after recrystallization from chloroform–hexane), lit. (Sheehan and Corey, 1952) mp 87–89°. Similarly, cyanomethyl hippurate was prepared from sodium hippurate and chloroacetonitrile, mp 100° (after recrystallization from chloroform–hexane). *Anal.*[2] Calcd for $C_{11}H_{10}N_2O_3$: C, 60.55; H, 4.62; N, 12.84. Found: C, 60.36; H, 4.90; N, 12.99. Methyl hippurate (Mann Research Laboratories), recrystallized from ethanol–water, had mp 81° and lit. (Lowe and Williams, 1965) mp 81°.

PO was prepared according to Crawford and Little (1959) and purified by vacuum sublimation which gave

* From the Department of Biochemistry, University of Queensland, St. Lucia, Queensland 4067, Australia. *Received October 24, 1968.* This work was supported in part by the A. R. G. C. (Australia) and Grant GM 13759 from the Institute of General Medical Sciences of the U. S. National Institutes of Health. Preliminary communications: de Jersey *et al.* (1966a,b).

† C. S. I. R. O. postgraduate student.

[1] Abbreviations used: PNPH, *p*-nitrophenyl hippurate; PO, 2-phenyloxazolin-5-one.

[2] Microanalyses were performed by the Australian Microanalytical Service, Melbourne, Victoria.

TABLE I: Characteristics of Acylamino Acid Nitrophenyl Esters.

Acylaminoacyl Group	Nitrophenol	Mp (°C)	Formula	Calcd (%)			Found (%)			% Purity[a]
				C	H	N	C	H	N	
Hippuryl	*p*-Nitrophenol	170[b]								99
Hippuryl	*m*-Nitrophenol	120[c]								98
Hippuryl	*o*-Nitrophenol	108–110	$C_{15}H_{12}N_2O_5$	60.00	4.03	9.33	59.45	4.20	9.44	98
Hippuryl	2,4-Dinitrophenol	101								98
Hippuryl	2,5-Dinitrophenol	117	$C_{15}H_{11}N_3O_7$	52.18	3.21	12.17	52.09	3.53	12.28	100
Hippuryl	*p*-Nitrothiophenol	166	$C_{15}H_{12}N_2O_4S$	56.95	3.83	8.85	56.94	4.12	9.03[f]	
Formylglycyl	*p*-Nitrophenol	139	$C_9H_8N_2O_5$	48.22	3.60	12.50	47.96	3.63	12.21	98
Acetylglycyl	*p*-Nitrophenol	126[d]								100
Cinnamoylglycyl	*p*-Nitrophenol	176–8	$C_{17}H_{14}N_2O_5$	62.57	4.32	8.58	62.30	4.52	8.80	99
Benzyloxycarbonylglycyl	*p*-Nitrophenol	127[e]								
Benzoylalanyl	*p*-Nitrophenol	145	$C_{16}H_{14}N_2O_5$	61.14	4.49	8.91	61.04	4.58	9.06	101
Benzoylaminoisobutyryl	*p*-Nitrophenol	115	$C_{17}H_{16}N_2O_5$	62.19	4.91	8.53	61.80	5.02	8.69	101
Benzoylsarcosyl	*p*-Nitrophenol	113	$C_{16}H_{14}N_2O_5$	61.14	4.49	8.91	61.32	4.67	9.13	100

[a] Based on the amount of nitrophenol released (measured spectrophotometrically) on complete hydrolysis of a known weight of ester, compared with the theoretical amount of nitrophenol from the same weight of pure ester. [b] Lit. (McDonald and Balls, 1957) mp 171°. [c] Lit. (Lowe and Williams, 1965) mp 118–122°. [d] Lit. (Lorand *et al.*, 1962) mp 116–118°. [e] Lit. (Kirsch and Igelström, 1966) mp 127–128°. [f] *Anal. S: Calcd*: 10.14. *Found*: 10.08.

colorless crystals, mp 90–90.5°. 4, 4-Dimethyl-2-phenyloxazolin-5-one was prepared by the action of dicyclohexylcarbodiimide on *N*-benzoylaminoisobutyric acid in ethyl acetate and purified by sublimation *in vacuo:* mp 46°, lit. (Mohr and Geis, 1908) mp 34°. Similarly, 4,4-dimethyl-2-styryloxazolin-5-one and 4,4-dimethyloxazolin-5-one were prepared from *N-trans*-cinnamoylaminoisobutyric acid and *N*-formylaminoisobutyric acid, and purified by vacuum sublimation. 4,4-Dimethyl-2-styryloxazolin-5-one melted sharply at 58°, but 4,4-dimethyloxazolin-5-one remained an oily solid after sublimation at 70°. 2-Methyl-4,4-dimethyloxazolin-5-one, prepared from *N*-acetylaminoisobutyric acid by reaction with dicyclohexylcarbodiimide, was a liquid showing a typical oxazolinone infrared absorption spectrum with intense peaks at 1810 and 1680 cm^{-1}. 4-Methyl-2-phenyloxazolin-5-one was prepared by the dicyclohexylcarbodiimide method and purified by sublimation *in vacuo* at 35°: mp 41°, lit. (Mohr and Stroschein, 1909) mp 39°. The 4-unsubstituted oxazolinones proved more difficult to isolate. The oxazolinones corresponding to *N*-formylglycine, *N*-acetylglycine, and *N-trans*-cinnamoylglycine were prepared by reacting the parent acylamino acids with an equimolar amount of dicyclohexylcarbodiimide in acetonitrile, filtering the dicyclohexylurea, and diluting to a suitable concentration for study of the oxazolinones *in situ*.

Buffers were prepared using analytical grade reagents. pH measurements were made on a Radiometer pH meter 4c, standardized according to Bates (1964), and are accurate to ±0.01 pH unit. 2,6-Lutidine was purified by preparation and repeated crystallization of the urea clathrate (Biddiscombe *et al.*, 1954), followed by fractionation on a 60-cm column packed with glass helices, bp 144°. Imidazole was recrystallized from benzene before use. Acetonitrile (Eastman Spectro Grade) was used without further purification. α-Chymotrypsin (three-times crystallized) was obtained from Worthington Biochemical Corp. *p*-Nitrophenyl acetate (Aldrich Chemical Co.) was recrystallized from chloroform–hexane.

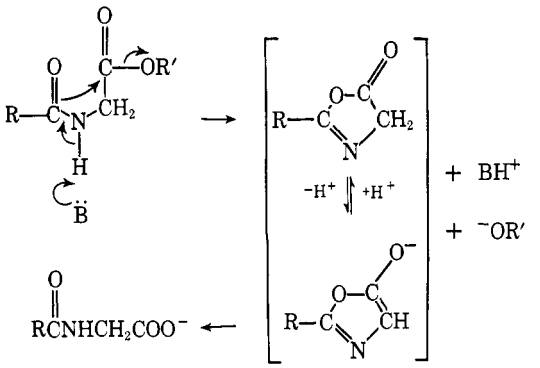
Methods. All kinetic experiments were carried out on a Cary 14 recording spectrophotometer, equipped with 0–0.1- and 0–1.0-absorbance slide wires and a thermostated cell compartment. The early experiments on the hydrolysis of PNPH were carried out at 30 ± 0.1° (de Jersey *et al.*, 1966a), while all other experiments were carried out at 25 ± 0.1°. In a typical experiment, 3 ml of buffer was equilibrated in the cell compartment, and the reaction was started by the addition of a small aliquot (usually 50 μl) of a solution of the substrate in acetonitrile on a flat-ended stirring rod. Ultraviolet absorption spectra of PO were determined at neutral pH and in 0.1 N NaOH. The neutral form of PO was sufficiently stable to permit determination of its spectrum by rapid scanning immediately after addition of the PO to buffer at pH 5. In 0.1 N NaOH, however, PO is very unstable ($t_{1/2} \simeq 20$ sec at 25°). The spectrum was determined by following the hydrolysis of PO in 0.1 N NaOH at a number of wavelengths and extrapolating the linear first-order plots thus obtained

to zero time. In this way, an accurate measure of A_0 and hence of ϵ at the particular wavelength was obtained. The conditions used for the determination of the alkaline rate constants for the various acylamino acid esters are given in the appropriate tables. When the rate constants were determined in 0.01 or 0.001 N NaOH, the alkaline rate constant for *p*-nitrophenyl acetate was determined at the same time under the same conditions. The observed rate constants were then corrected, using a value of 12.6 M^{-1} sec^{-1} for the alkaline rate constant of *p*-nitrophenyl acetate (K. A. Connors, 1961, personal communication).

Results

The Hydrolysis of PNPH. Based on the release of *p*-nitrophenol (measured at 317 or 400 mμ, depending upon the pH), PNPH has the abnormally high apparent alkaline rate constant, k_{OH}, of 1.05 × 10^4 M^{-1} sec^{-1} at 30° (Figure 1). When the hydrolysis of PNPH is observed at alkaline pH and 340 mμ, a rapid increase in absorbance (on a time scale corresponding to the release of *p*-nitrophenol) is followed by a first-order decrease in absorbance (Figure 2). Above pH 8.5, the first-order rate constant for the decay of the intermediate can be determined without interference from the release of *p*-nitrophenol. The following mechanism

SCHEME I



(Scheme I) was postulated to explain the observations, and is supported by the following results (for PNPH, R = phenyl, R′ = *p*-nitrophenyl). (1) In the proposed mechanism, the observed intermediate is identified with PO. Rate constants for the hydrolysis of the intermediate and independently synthesized PO were determined under a wide variety of conditions from pH 7.5 to 12.7. Identical rate constants for the decay of the intermediate and of PO were observed (de Jersey *et al.*, 1966a).[3] (2) The first step in the proposed scheme is general base catalyzed. Therefore, the hydrolysis of PNPH was studied in 2,6-lutidine buffers. Significant catalysis of *p*-nitrophenol release from PNPH by 2,6-lutidine was observed, but catalysis was not observed with the *N*-methyl analog, *N*-benzoylsarcosine *p*-nitrophenyl ester, or with *p*-nitrophenyl acetate

[3] The isosbestic point of *p*-nitrophenyl hippurate and *p*-nitrophenol–*p*-nitrophenolate ion at pH 7.5 is 306 mμ, not 360 mμ as previously listed in error (de Jersey *et al.*, 1966a).

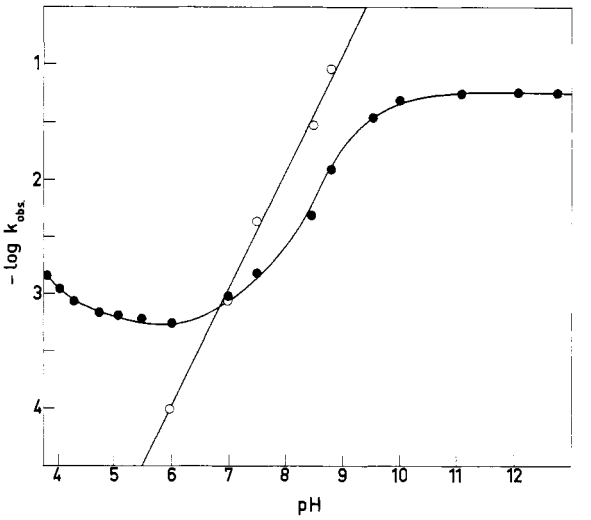


FIGURE 1: pH–rate profiles of *p*-nitrophenyl hippurate (○—○—○), as determined by release of *p*-nitrophenol, and of 2-phenyloxazolin-5-one (●—●—●) at 30°. Units of k_{obsd}, sec^{-1}; 1–3% (v/v) acetonitrile in reaction mixtures. k_{obsd} was measured in buffers of constant pH and increasing buffer concentration, and the k_{obsd} at zero buffer concentration was determined by extrapolation.

(de Jersey *et al.*, 1966a). (3) Figure 1 shows pH–rate profiles for the release of *p*-nitrophenol from PNPH and for the hydrolysis of PO. At pH 11, the release of *p*-nitrophenol is virtually instantaneous while the intermediate has a half-life of about 14 sec at 30°. PNPH (2 ml of 2 × 10^{-2} M solution in acetonitrile) was added rapidly to 200 ml of vigorously stirred 0.001 N NaOH at room temperature. After about 2 sec, 50 ml of 0.1 M acetate buffer (pH 5.1) was added, since at pH 5, PO is relatively stable. The solution was extracted immediately with chloroform, and *p*-nitrophenol was removed from the chloroform solution by exhaustive extraction with phosphate buffer (pH 7.6). After drying the solution over Na_2SO_4, its ultraviolet absorption spectrum was determined, and it proved to be very similar to the spectrum of an authentic sample of PO in chloroform (pronounced shoulder at 285 mμ). The procedure was repeated several times, the chloroform removed at reduced pressure, and the oily residue purified by vacuum sublimation. The infrared spectrum of the sublimed material was identical with that of PO run under the same conditions. (4) Analysis of the pH–rate profile for the intermediate at high pH (Figure 1) yields an apparent pK_a of 9.3. The effect of pH on the ultraviolet absorption spectrum of PO was determined. In aqueous buffers below pH 8, λ_{max} was found to be 240 mμ, while in 0.1 N NaOH, λ_{max} was 340 mμ. The apparent pK_a for this spectral change, determined by spectrophotometric titration at 340 mμ, is 9.4.

To determine whether the hydrolysis of PNPH proceeds quantitatively through PO, an aliquot of PNPH was added to 0.1 N NaOH and the absorbance at 340 mμ followed. Extrapolation of the absorbance to zero time, using a linear first-order plot for accuracy, gives a value for ΔA at 340 mμ. Independent experiments give a value of 15,200 for $\Delta\epsilon$ at 340 mμ for the

TABLE II: Hydrolysis of Acylglycine *p*-Nitrophenyl Esters at 25°. Effect of the Acyl Group, R_1.

R_1	k_{OH}[a] (M^{-1} sec^{-1})	Wavelength[b] ($m\mu$)	Intermediate 10^2k_{obsd}[c] (sec^{-1})	Oxazolinone 10^2k_{obsd}[c] (sec^{-1})
Benzoyl	7040[d]	340	3.46	3.46
Cinnamoyl	3480[e]	390	2.61	2.58
Acetyl	1110[d]	220		1.16
Formyl	480[d]	250	6.57	6.57
Benzyloxycarbonyl	156[f]			
p-Toluenesulfonyl[g]	38[f]			

[a] Apparent alkaline rate constants for release of *p*-nitrophenol at 400 $m\mu$; $pK_w = 14$. [b] Wavelength at which the intermediate was observed. [c] Measured in 0.1 N NaOH. [d] k_{obsd} was determined in a series of sodium borate buffers (pH 9.2). The k_{obsd} at zero borate concentration was determined by extrapolation and converted to k_{OH}. [e] Determined in Tris buffers (pH 8.14) at 410 $m\mu$. [f] Measured in 0.001 N NaOH. [g] Impure tosylglycine *p*-nitrophenyl ester, prepared from tosylglycine and *p*-nitrophenol coupled with dicyclohexylcarbodiimide.

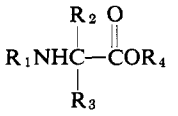
TABLE III: Hydrolysis of Acylamino Acid Esters at 25°. Variation of R_2 and R_3.[a]

R_2	R_3	k_{OH}[b] (M^{-1} sec^{-1})	Intermediate 10^2k_{obsd} (sec^{-1})	Oxazolinone 10^2k_{obsd} (sec^{-1})
H	H	7,040[c]	3.46[f]	3.46[f]
CH_3	H	59,400[d]	1.91[g]	1.91[g]
CH_3	CH_3	346,000[e]	0.101[h]	0.099[h]

[a] R_1 = benzoyl; R_4 = *p*-nitrophenyl. [b] Rate of release of *p*-nitrophenol (400 $m\mu$). [c] Determined in sodium borate buffers (pH 9.2) and extrapolated to zero borate concentration. [d] Determined in Tris buffers (pH 8.10). [e] Determined in phosphate buffers (pH 7.16). [f] In 0.1 N NaOH; measured at 340 $m\mu$. [g] In 0.1 N NaOH; measured at 350 $m\mu$. [h] In 0.05 M Tris buffer (pH 8.14); measured at 250 $m\mu$.

hydrolysis of PO in 0.1 N NaOH. From ΔA and $\Delta\epsilon$, the concentration of PO formed from a known concentration of PNPH could be calculated. Results established the quantitative conversion of PNPH to PO. Similarly, the hydrolysis of PNPH in 0.05 M barbital buffer (pH 9.15) was followed at 340 $m\mu$. The progress curve observed is shown in Figure 2. The rate constants for the release of *p*-nitrophenol from PNPH (determined at 400 $m\mu$ where PO does not absorb) and for the hydrolysis of PO were 1.07×10^{-1} and 1.18×10^{-2} sec^{-1}, respectively. The apparent molar absorptivities at 340 $m\mu$ of the four species present were determined independently under the conditions of the experiment: $\epsilon_{p\text{-nitrophenol}}$ 3950, ϵ_{PNPH} 720, ϵ_{PO} 4610, and $\epsilon_{hippuric\ acid} \leq 10$. From these data, the theoretical curve assuming quantitative formation and hydrolysis of PO was calculated. Results indicate that at least 90% of the theoretical amount of PO is formed. At pH 9.15, two molecules of PO can react to form a product which absorbs at 340 $m\mu$, and which is stable at pH 9. This condensation reaction, which occurs maximally near the pK_a of PO (9.4), competes with the hydrolytic reaction and probably accounts for the discrepancy between the observed and calculated progress curves.

The Generality of the Oxazolinone Mechanism. A series of esters of *N*-acylamino acids was synthesized to test the effect of variation of substituent groups on the mechanism of hydrolysis. Consider the general formula for an acylamino acid ester



R_1 is the acyl group, R_2 and R_3 are substituents on the α-carbon, and $^-OR_4$ is the leaving group. Two general criteria were used to determine whether an oxazolinone intermediate is formed in the hydrolysis of a particular ester. The main criterion was the spectrophotometric observation of the intermediate and the comparison of its rate of hydrolysis and spectral properties with those of the independently synthesized oxazolinone. The second criterion was the rate of alkaline hydrolysis.

The importance of the acyl group was tested by examining a series of *N*-acylglycine *p*-nitrophenyl esters (Table II). For cinnamoyl and formyl derivatives, the oxazolinone intermediates were readily demonstrated. The oxazolinone corresponding to the acetyl derivative had very poor spectral characteristics ($\Delta\epsilon \sim 150$ at 220 $m\mu$ in 0.1 N NaOH), making observation of an inter-

TABLE IV: Hydrolysis of Esters of Hippuric Acid at 25°.

R_4	k_{OH} (M^{-1} sec^{-1})	Conditions of Measurement[a]	Wavelength (mμ)	2-Phenyloxazolin-5-one Formed[b] (%)
p-Nitrophenyl	7,040	Borate buffers, pH 9.20	400	100
o-Nitrophenyl	10,200	Borate buffers, pH 9.20	440	99
m-Nitrophenyl	1,640	Borate buffers, pH 9.20	400	97
p-Nitrothiophenyl	29,000	Tris buffers, pH 8.14	412	97
2,4-Dinitrophenyl	Too fast			100
2,5-Dinitrophenyl	340,000	Phosphate buffers, pH 7.16	450	102
Phenyl	(34)[c]	Borate buffers, pH 9.20	270	50
Cyanomethyl	78	0.001 N NaOH	250	0.35
Benzyl	2.2	0.01 N NaOH	250	0
Methyl	2.1	0.01 N NaOH	250	0

[a] k_{obsd} was determined in a series of buffers, and the value at zero buffer concentration was estimated by extrapolation. [b] Percentage 2-phenyloxazolin-5-one formed on hydrolysis of esters in 0.1 N NaOH; [2-phenyloxazolin-5-one] = ΔA at 340 mμ/15,200. [c] A value could not be obtained in 0.001 N NaOH because of the absorbance of 2-phenyloxazolin-5-one at 270 mμ. At pH 9.2, the formation, rather than the decomposition, of 2-phenyloxazolin-5-one would be rate limiting.

mediate exceedingly difficult. However, the high k_{OH} for *N*-acetylglycine *p*-nitrophenyl ester indicates that in all probability the oxazolinone mechanism applies. Because the oxazolinones corresponding to *N*-formyl- and *N-trans*-cinnamoylglycine were not isolated, accurate $\Delta\epsilon$ values were not obtained. Results strongly suggest that the oxazolinones are formed quantitatively from the *p*-nitrophenyl esters in 0.1 N NaOH. No intermediates could be observed in the hydrolysis of *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester or *N*-*p*-toluenesulfonylglycine *p*-nitrophenyl ester. The k_{OH}'s for these compounds are correspondingly low.

The hydrolysis of two α-substituted benzoylamino acid *p*-nitrophenyl esters was studied (Table III). The hydrolysis of *N*-benzoylaminoisobutyric *p*-nitrophenyl ester and *N*-benzoyl-DL-alanine *p*-nitrophenyl ester yielded 4,4-dimethyl-2-phenyloxazolin-5-one and 4,4-dimethyl-2-phenyloxazolin-5-one in yields of 100 and 117%, based on the measured $\Delta\epsilon$'s of the isolated oxazolinones. Thus, hydrolysis proceeds quantitatively through the oxazolinone in each case (assuming the isolated 4-methyl-2-phenyloxazolin-5-one is 85% pure). The effect of α substitution on the alkaline rate constants was also determined.

The hydrolysis of ten esters of hippuric acid has been observed in an attempt to define the leaving group requirements of the oxazolinone route (Table IV). All of the substituted phenyl esters examined yielded a quantitative amount of PO in 0.1 N NaOH. Phenyl hippurate gives 50% of the calculated amount of PO when the hydrolysis is carried out in 0.1 N NaOH (55%, if 1 N NaOH is used). Cyanomethyl hippurate gives a trace of PO. The alkaline rate constant of 2.1 M^{-1} sec^{-1} for the hydrolysis of methyl hippurate, determined spectrophotometrically, may be compared with a rate constant of 1.7 M^{-1} sec^{-1} determined titrimetrically (Hay and Morris, 1967).

The Deacylation of Hippuryl-chymotrypsin. Hippuryl-chymotrypsin was prepared from α-chymotrypsin and PNPH at pH 2.9, with enzyme present in a two-fold molar excess. The acylation reaction was followed to infinity at 317 mμ (release of *p*-nitrophenol). Aliquots of acyl-enzyme were then added to 0.1 M sodium borate buffer (pH 9.2) and 0.1 N NaOH and the reactions observed at 340 mμ. In neither case was there an appreciable change in absorbance with time, indicating that no PO was formed. The sensitivity of the method was such that 2% of the theoretical amount of PO could have been detected. In control experiments, the rate of deacylation of hippuryl-chymotrypsin was shown to be negligible at pH 2.9. Titration with *N-trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961) before and after addition of PNPH showed that an amount of hippuryl-chymotrypsin equal to the amount of PNPH added had been formed.

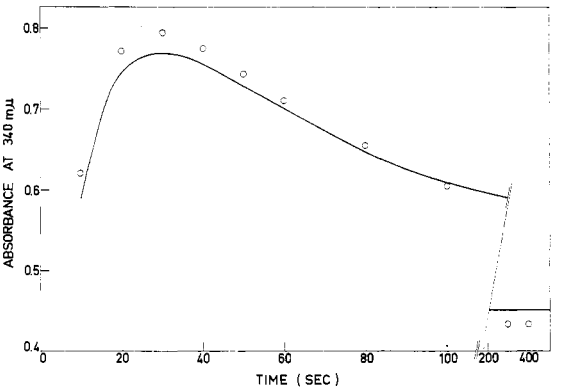


FIGURE 2: Progress curve for the hydrolysis of *p*-nitrophenyl hippurate in 0.05 M barbital buffer (pH 9.15) at 25°: solid curve, experimental trace; open circles, points calculated using rate constants and apparent molar absorptivities independently determined (see text).

The Effect of Imidazole on the Hydrolysis of Acylamino Acid Esters. The kinetics of hydrolysis of PNPH, *N*-benzoyl-DL-alanine *p*-nitrophenyl ester, and *N*-

TABLE V: Catalysis by Imidazole and Phosphate Ion of the Hydrolysis of Acylamino Acid Esters at 25°.

Buffer	Concn (M)[a]	pH	k_{obsd} *p*-Nitrophenyl Hippurate (sec^{-1})	k_{obsd} *N*-Benzoyl-DL-alanine *p*-Nitrophenyl Ester[b] (sec^{-1})	k_{obsd} *N*-Benzoylamino-isobutyric *p*-Nitrophenyl[b] Ester (sec^{-1})
Imidazole	0.20	7.05	1.55×10^{-1}	1.73×10^{-1}	3.98×10^{-2}
Imidazole	0.10	7.07	7.75×10^{-2}	8.69×10^{-2}	3.95×10^{-2}
Imidazole	0.05	7.05	3.85×10^{-2}	4.77×10^{-2}	3.83×10^{-2}
Imidazole	0.025	7.06	1.93×10^{-2}	2.83×10^{-2}	3.87×10^{-2}
Imidazole	0.01	7.05	7.83×10^{-3}	1.62×10^{-2}	3.80×10^{-2}
Phosphate	0.01667	7.14	1.21×10^{-3}	1.05×10^{-2}	
Phosphate	0.012	7.11	1.06×10^{-3}	9.8×10^{-3}	
Phosphate	0.010	7.08	1.01×10^{-3}	9.3×10^{-3}	
Phosphate	0.005	7.04	0.91×10^{-3}	8.7×10^{-3}	

[a] For imidazole buffers, μ was kept constant at 0.1 with KCl. [b] Measured by following release of *p*-nitrophenol at 400 mμ.

benzoylaminoisobutyric *p*-nitrophenyl ester in imidazole and phosphate buffers at pH 7 were studied by measuring the rate of release of *p*-nitrophenol (Table V). The catalytic rate constants for imidazole, $k_{imidazole}$, in the hydrolysis of PNPH and *N*-benzoyl-DL-alanine *p*-nitrophenyl ester were both 1.56 M^{-1} sec^{-1}. For both esters, the catalytic rate constant for phosphate dianion, k_P, was small. After correcting for the pH differences in the phosphate buffers, k_P was estimated at <0.2 M^{-1} sec^{-1} for *N*-benzoyl-DL-alanine *p*-nitrophenyl ester and <0.05 M^{-1} sec^{-1} for PNPH. Both these figures must be regarded as approximate only. $k_{imidazole}$ in the hydrolysis of *N*-benzoylaminoisobutyric *p*-nitrophenyl ester was estimated at $\sim 10^{-2}$ M^{-1} sec^{-1}. When the reaction of PNPH with imidazole is followed at 250 mμ, the formation and decay of an intermediate are observed. PO reacts with imidazole to form the same intermediate.

Discussion

A considerable amount of experimental evidence for the proposed mechanism of hydrolysis of PNPH has been obtained. The intermediate observed in the hydrolysis of PNPH has been identified as PO, on the basis of the identity of the rate constants for hydrolysis under various conditions (de Jersey *et al.*, 1966a) and by a comparison of ultraviolet absorption spectra. The intermediate has also been isolated and found to have ultraviolet and infrared spectra identical with those of PO run under the same conditions. The effect of steric hindrance on catalysis is one important criterion by which general base and nucleophilic catalysis are distinguished. 2,6-Lutidine has no detectable effect on the hydrolysis of acetic anhydride (Butler and Gold, 1961) or acetyl phosphate (di Sabato and Jencks, 1961). Catalysis of both these reactions by pyridine is nucleophilic, involving the formation and decay of acetylpyridinium ion. General base catalysis by 2,6-lutidine is also subject to steric hindrance, but to a lesser degree (Covitz and Westheimer, 1963). The observed catalysis of PNPH hydrolysis by 2,6-lutidine (de Jersey *et al.*, 1966a) is therefore evidence that the release of *p*-nitrophenol is indeed general base catalyzed as required by the mechanism. This conclusion is supported by the absence of catalysis of the hydrolysis of *N*-benzoylsarcosine *p*-nitrophenyl ester and *p*-nitrophenyl acetate by 2,6-lutidine.

The proposed equilibrium between neutral PO and its enolate ion (PO^-) explains the observed effect of pH on the hydrolysis of the intermediate. If eq 1 applies, then k_{obsd} equals $k_1[PO][OH^-]$ or the kinetically equivalent expression $k_2[PO^-][H_2O]$. If we assume that PO^- is resistant to attack by OH^-, then at the pK_a, the observed rate constant is half the maximum rate constant. At pH values below 8, PO has λ_{max} 240 mμ, which agrees well with the λ_{max} measured in ether (Cornforth, 1949), indicating that PO is uncharged at neutral pH. In our formulation, the species with λ_{max} 340 mμ is PO^-. The observed spectral shift is therefore explained by the extended conjugation of the enolate ion. The implications of this equilibrium for the mechanism of racemization of oxazolinones are discussed in the following paper (de Jersey and Zerner, 1969).

$$\begin{array}{ccc} PO & \overset{K_a}{\rightleftharpoons} & PO^- + H^+ \\ {\scriptstyle k_1}\downarrow {\scriptstyle -OH} & & {\scriptstyle k_2}\downarrow {\scriptstyle H_2O} \\ \text{hippuric acid} & & \end{array} \qquad (1)$$

The results obtained with a series of *N*-substituted glycine *p*-nitrophenyl esters (Table II) indicate that for formyl, acetyl, and cinnamoyl derivatives, an oxazolinone is formed as the first step in the hydrolysis. The *N*-acyl group has a large effect on the rate of formation of oxazolinone as measured by the apparent alkaline rate constants. The order of decreasing k_{OH}'s (benzoyl > acetyl > formyl) is the same as the order of decreasing

susceptibility of *N*-acylamino acids to racemization during peptide synthesis. Antonovics *et al.* (1966) determined the effect of the acyl group on the extent of racemization occurring in the reaction

$$\text{acyl-L-leucine} + \text{glycine ethyl ester} \xrightarrow{\text{DCC}-CH_2Cl_2} \text{acyl-L-leucylglycine ethyl ester}$$

Benzoyl, acetyl, and formyl derivatives gave 53, 70, and 94%, respectively, of the L product. Together, these two results heavily indicate that the rate-limiting step in the racemization of acylamino acid derivatives during peptide synthesis is the formation of the oxazolinone. Benzyloxycarbonyl and *p*-toluenesulfonyl substituents prevent oxazolinone formation during hydrolysis. Similarly, they minimize racemization in peptide synthesis. Variation of the degree of substitution at the α-carbon has a marked effect on the rate of oxazolinone formation (Table III). k_{OH} for *N*-benzoyl-aminoisobutyric *p*-nitrophenyl ester, with *gem*-dimethyl substitution, is greater than k_{OH} for PNPH, where the α-carbon is unsubstituted, by a factor of 50. Bruice and Pandit (1960) found a similar effect of *gem*-dimethyl substitution in the hydrolyses of monoglutarate esters, which proceed by formation and hydrolysis of the anhydride. *N*-Benzoyl-DL-alanine *p*-nitrophenyl ester, with one methyl substituent at the α-carbon, shows intermediate behavior. Similarly all other benzoyl-amino acid *p*-nitrophenyl esters should yield the corresponding oxazolinones faster than does PNPH.

The nature of the leaving group has a profound effect on the mechanism of ester hydrolysis. For example, in the hydrolysis of phenyl hydrogen phthalate, nucleophilic attack by the carboxylate ion leads to formation of phthalic anhydride (Thanassi and Bruice, 1966). However, the hydrolysis of methyl hydrogen phthalate is catalyzed intramolecularly by the undissociated carboxyl group rather than by the anion. From the results obtained with a series of hippurate esters (Table IV), a correlation between the pK_a' of the leaving group and the percentage of oxazolinone formed can be made. For the substituted phenyl esters examined, the oxazolinone intermediate is formed quantitatively, and so the k_{OH} values give a measure of the effects of the substituents. Steric hindrance at the ester carbonyl is not important in oxazolinone formation (Scheme I), and the k_{OH} values therefore reflect the inductive effects of the substituents. The ratio of k_{OH}'s obtained for *o*-, *m*- and *p*-nitro groups (1.45:0.23:1) is therefore of interest, in that the value obtained for the *o*-nitro group (*vis-à-vis* the *para*) is uncomplicated by steric hindrance. Two leaving groups (phenoxide and cyanomethoxide) give intermediate values of PO, defining the degree of activation of the leaving group necessary for oxazolinone formation in this system (0.1 N NaOH). Stable esters such as methyl and benzyl hippurate give no oxazolinone under the conditions used.

Goodman and Stueben (1962) obtained considerable indirect evidence for the formation of an oxazolinone which they postulated to account for the racemization observed during the hydrolysis of concentrated solutions of benzyloxycarbonylglycyl-L-phenylalanine *p*-nitrophenyl ester in 64% dioxane–buffer mixtures. However, no evidence for the mechanism of hydrolysis of the ester was obtained, and Goodman and Stueben appear to have argued against hydrolysis *via* oxazolinone as the major route in this system. Other workers (Williams and Young, 1964; Antonovics and Young, 1965; Goodman and Levine, 1964) have produced additional evidence for equilibria between *N*-acylamino acid *p*-nitrophenyl esters and their corresponding oxazolinones in organic solvents in the presence of various bases. Our results indicate that in aqueous alkaline solution, the equilibrium position is overwhelmingly in favor of the oxazolinone, and that the hydrolysis of activated esters proceeds quantitatively through the corresponding oxazolinones. Kemp and Chien (1967) obtained evidence for the existence of an intermediate between acylamino acid ester and oxazolinone in *N,N*-dimethylformamide containing triethylamine and its fluoroborate salt. The present results in aqueous solution do not comment on this intermediate.

Peptide activated esters are important intermediates in peptide synthesis. Recently, peptide derivatives of *N*-hydroxyphthalimide (Nefkens and Tesser, 1961), *N*-hydroxysuccinimide (Anderson *et al.*, 1963), and *N*-hydroxypiperidine (Beaumont *et al.*, 1965) have been introduced as activated esters. These intermediates undergo coupling with amino acid esters without racemization. Further, they are readily synthesized without racemization, by contrast with peptide *p*-nitrophenyl esters which are exceedingly difficult to obtain optically pure by direct methods (Anderson *et al.*, 1967; Zimmerman and Anderson, 1967). Goodman and Glaser (1968) have proposed that such *N*-hydroxyimides do not cause racemization because of their 1,2-dinucleophile structure.

Goodman and Glaser (1968) also observed that diethylhydroxylamine will open an oxazolinone ring rapidly without racemization. However, dimethylhydrazine opens the oxazolinone ring more slowly and with complete racemization. To explain these data, they have postulated that the special properties of certain 1,2-dinucleophiles depend on their ability to form hydrogen-bonded five-membered rings with the carbonyl group of the oxazolinone. The zwitterion of diethylhydroxylamine ($Et_2HN^+O^-$) could form such a five-membered ring, the initial product being an *O*-acylhydroxylamine. Dimethylhydrazine, on the other hand, could not form the required product-producing hydrogen-bonded complex. It seems simpler, however, to propose that the nucleophilicity of dimethylhydrazine is not enhanced with respect to its basicity. Indeed, this proposal is adequately supported by the study of Jencks and Carriuolo (1960) of the effect of numerous nucleophiles on the hydrolysis of *p*-nitrophenyl acetate. Dimethylhydroxylamine, hydroxylamine, and hydrazine show large positive deviations from the Brønsted plot, while dimethylhydrazine shows a smaller negative deviation. Therefore, in the absence of further and more compelling evidence, the concept of the hydrogen-bonded complex seems unnecessary. It would be of

interest in this connection to determine whether the hydroxyimide derivatives of hippuric acid yield PO in 0.1 N NaOH.

The deacylation rate constant of hippuryl-chymotrypsin in 0.1 M phosphate buffer (pH 6.97) at 25° is 0.53 sec^{-1} (de Jersey *et al.*, 1966b). Therefore, if the acyl-enzyme is a serine ester, it is a very reactive one. The deacylation reaction is currently held to be catalyzed by imidazole acting as a general base (Bender and Kézdy, 1965). Since the formation of oxazolinone from activated acylamino acid esters is general base catalyzed, it was postulated that a suitably positioned imidazole residue could catalyze the formation of PO from hippuryl-chymotrypsin. However, no evidence for oxazolinone formation was obtained. Therefore, although hippuryl-chymotrypsin is a very reactive ester, it is not an activated ester in terms of the oxazolinone mechanism. In other words, the high reactivity of hippuryl-chymotrypsin does not seem to be due to activation of the serine leaving group. Further evidence that deacylation of acyl-enzymes does not occur *via* oxazolinone intermediates comes from specificity studies. Benzyloxycarbonylamino acid esters compare favorably with benzoylamino acid esters as substrates for α-chymotrypsin and other proteolytic enzymes. Similarly, *p*-toluenesulfonyl-L-arginine methyl ester is as good a substrate for trypsin as benzoyl-L-arginine methyl ester (Schwert *et al.*, 1948).

The ratio of $k_{imidazole}/k_P$ has been used to distinguish between nucleophilic and general base catalysis (Johnson, 1967). The ratios determined for PNPH (~300) and *N*-benzoyl-DL-alanine *p*-nitrophenyl ester (~75) are in the range expected for nucleophilic catalysis (20–5000). Further evidence that imidazole reacts as a nucleophile with PNPH was obtained from the observation of an intermediate, hippurylimidazole. The $k_{imidazole}$ estimated in *N*-benzoylaminoisobutyric *p*-nitrophenyl ester hydrolysis is less than that in PNPH hydrolysis by a factor of 150. This factor could be caused by steric hindrance to the formation of an acylimidazole intermediate (Zerner and Bender, 1961). No such intermediate could be detected in the reaction of 4,4-dimethyl-2-phenyloxazolin-5-one with imidazole. Staab (1956) measured the rates of hydrolysis of a number of acylimidazoles in conductivity water. Trimethylacetylimidazole hydrolyzed 11 times faster than acetylimidazole. This indicates that the formation of benzoylaminoisobutyrylimidazole, rather than its hydrolysis, would be rate limiting. It seems likely, therefore, that imidazole may act as a nucleophile in the hydrolysis of all three esters. In the hydrolysis of PNPH, nucleophilic catalysis by imidazole (to give hippurylimidazole) is clearly favored over general base catalysis by imidazole (to give PO).

Acknowledgments

We acknowledge with thanks the indefinite loan of a Cary 14 recording spectrophotometer from the Wellcome Trust (London), and thank Mr. A. A. Kortt, Dr. R. L. Blakeley, and Dr. J. K. Stoops for the synthesis of some of the compounds used.

References

Anderson, G. W., Callahan, F. M., and Zimmerman, J. E. (1967), *J. Amer. Chem. Soc. 89*, 178.

Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1963), *J. Amer. Chem. Soc. 85*, 3039.

Antonovics, I., Heard, A. L., Hugo, J., Williams, M. W., and Young, G. T. (1966), *in* Proceedings of 6th European Symposium, Zervas, L., Ed., Oxford, Pergamon, p 121.

Antonovics, I., and Young, G. T. (1965), *Chem. Commun.*, 398.

Bates, R. G. (1964), Determination of pH. Theory and Practice, New York, N. Y., Wiley.

Beaumont, S. M., Hanford, B. O., Jones, J. H., and Young, G. T. (1965), *Chem. Commun.*, 53.

Bender, M. L. (1960), *Chem. Rev. 60*, 53.

Bender, M. L., and Kézdy, F. J. (1965), *Ann. Rev. Biochem. 34*, 49.

Biddiscombe, D. P., Coulson, E. A., Handley, R., and Herington, E. F. (1954), *J. Chem. Soc.*, 1957.

Bruice, T. C., and Pandit, U. K. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 402.

Butler, A. R., and Gold, V. (1961), *J. Chem. Soc.*, 4362.

Cornforth, J. W. (1949), *in* The Chemistry and Penicillin, Princeton, N. J., Princeton University, p 758.

Covitz, F., and Westheimer, F. H. (1963), *J. Amer. Chem. Soc. 85*, 1773.

Crawford, M., and Little, W. T. (1959), *J. Chem. Soc.*, 729.

de Jersey, J., Kortt, A. A., and Zerner, B. (1966a), *Biochem. Biophys. Res. Commun. 23*, 745.

de Jersey, J., Runnegar, M. T. C., and Zerner, B. (1966b), *Biochem. Biophys. Res. Commun. 25*, 383.

de Jersey, J., and Zerner, B. (1969), *Biochemistry 8*, 1967 (this issue; paper 2).

di Sabato, G., and Jencks, W. P. (1961), *J. Amer. Chem. Soc. 83*, 4393.

Goodman, M., and Glaser, C. (1968), *Chem. Eng. News 46* (13), 40.

Goodman, M., and Levine, L. (1964), *J. Amer. Chem. Soc. 86*, 2918.

Goodman, M., and Stueben, K. C. (1962), *J. Org. Chem. 27*, 3409.

Hay, R. W., and Morris, P. J. (1967), *Chem. Commun.*, 663.

Jencks, W. P., and Carriuolo, J. (1960), *J. Amer. Chem. Soc. 82*, 1778.

Johnson, S. L. (1967), *Advan. Phys. Org. Chem. 5*, 237.

Kemp, D. S., and Chien, S. W. (1967), *J. Amer. Chem. Soc. 89*, 2745.

Kirsch, J. F., and Igelström, M. (1966), *Biochemistry 5*, 783.

Lorand, L., Brannen, W., and Rule, N. (1962), *Arch. Biochem. Biophys. 96*, 147.

Lowe, G., and Williams, A. (1965), *Biochem. J. 96*, 199.

McDonald, C. E., and Balls, A. K. (1957), *J. Biol. Chem. 227*, 727.

Mohr, E., and Geis, T. (1908), *Chem. Ber. 41*, 798.

Mohr, E., and Stroschein, H. (1909), *Chem. Ber. 42*, 2521.
Nefkens, G. H. L., and Tesser, G. I. (1961), *J. Amer. Chem. Soc. 83*, 1263.
Schwert, G. W., Neurath, H., Kaufman, S., and Snoke, J. E. (1948), *J. Biol. Chem. 172*, 221.
Schonbaum, G. R., Zerner, B., and Bender, M. L. (1961), *J. Biol. Chem. 236*, 2930.
Sheehan, J. C., and Corey, E. J. (1952), *J. Amer. Chem. Soc. 74*, 4555.
Staab, H. A. (1956), *Chem. Ber. 89*, 2088.
Thanassi, J., and Bruice, T. C. (1966), *J. Amer. Chem. Soc. 88*, 747.
Weiss, F. (1893), *Chem. Ber. 26*, 1700.
Williams, M. W., and Young, G. T. (1964), *J. Chem. Soc.*, 3701.
Zerner, B., and Bender, M. L. (1961), *J. Amer. Chem. Soc. 83*, 2267.
Zimmerman, J. E., and Anderson, G. W. (1967), *J. Amer. Chem. Soc. 89*, 7151.

On the Spontaneous and Enzyme-Catalyzed Hydrolysis of Saturated Oxazolinones*

John de Jersey† and Burt Zerner

ABSTRACT: The spontaneous and enzyme-catalyzed hydrolyses of a number of saturated oxazolinones have been investigated by following the decrease in ultraviolet absorbance which occurs on ring opening. Rate constants determined for the hydrolyses of saturated oxazolinones indicate the high reactivity of the oxazolinone carbonyl carbon toward nucleophilic attack. Oxazolinones have been shown to be good substrates for a number of hydrolytic enzymes. 2-Phenyloxazolin-5-one and 4,4-dimethyl-2-phenyloxazolin-5-one react rapidly with α-chymotrypsin, trypsin, and papain, forming relatively stable acyl-enzymes. The acylation reaction may be observed directly, providing evidence for the three-step mechanism of hydrolysis and a method for titration of the enzyme active sites. 2-Phenyloxazolin-5-one and *p*-nitrophenyl hippurate have been compared as substrates for α-chymotrypsin and ox liver carboxylesterase. Kinetic data obtained for the α-chymotrypsin-catalyzed hydrolysis of DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one provide an estimate of the optical specificity shown in both acylation and deacylation reactions.

Oxazolinones have been used as intermediates in a wide variety of organic syntheses (Carter, 1946). Bergmann *et al.* (1926) used oxazolinones as intermediates in peptide synthesis, and Leplawy *et al.* (1960) showed that oxazolinones are useful intermediates in the synthesis of peptides containing α-aminoisobutyric acid. The thiazolidine–oxazolinone hypothesis for the structure of penicillin inspired intensive research into the synthesis and reactions of saturated oxazolinones (Cornforth, 1949), a subject which had been largely neglected since the first saturated oxazolinone was isolated by Mohr and Geis (1908). Saturated oxazolinones were found to react readily with water, alcohols, and amines, reaction occurring at the carbonyl carbon, yielding the corresponding *N*-acylamino acid derivatives. Recently, it has been demonstrated that the transient formation of oxazolinones accounts for much of the racemization occurring during peptide synthesis (Williams and Young, 1964; Antonovics and Young, 1965). Optically pure oxazolinones have been prepared, and the relative rates of ring opening and racemization by various reagents have been determined (Goodman and Levine, 1964; Goodman and McGahren, 1967). However, no systematic, quantitative study of the behavior of saturated oxazolinones in aqueous solution has previously been reported.

Oxazolinones may be considered as activated internal esters of *N*-acylamino acids. Since activated esters of *N*-acylamino acids have been very useful in studies of the mechanism of action of proteolytic enzymes (Zerner and Bender, 1964), certain oxazolinones have been examined as substrates for several hydrolytic enzymes. Further, it has been shown that activated esters of *N*-acylamino acids hydrolyze through the corresponding oxazolinones (de Jersey *et al.*, 1969). It is therefore clearly important to determine the effect of the presence of oxazolinones on the kinetic constants determined for the enzyme-catalyzed hydrolyses of the corresponding activated esters.

In the present work, the spectral properties and rate constants for the spontaneous hydrolysis of several saturated oxazolinones have been determined. The reactions between certain oxazolinones and α-chymotrypsin, trypsin, papain, and ox liver carboxylesterase have been investigated. The implications of the results obtained for the mechanism of racemization of oxazolinones and the mechanism and specificity of hydrolytic enzymes are discussed.

* From the Department of Biochemistry, University of Queensland, St. Lucia, Queensland 4067, Australia. *Received October* 24, 1968. This work was supported in part by the A. R. G. C. (Australia) and Grant GM 13759 from the Institute of General Medical Sciences of the National Institutes of Health. Preliminary communications: de Jersey *et al.* (1966a,b).

† C. S. I. R. O. postgraduate student.