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.

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TABLE I: Spectral Properties and Dissociation Constants of Saturated Oxazolinones.

Oxazolinone	λ_{max}[a] (mμ) Neutral Species	λ_{max}[b] (mμ) Anion	pK_a'[c]
Oxazolin-5-one	<220	249	9.5
2-Methyloxazolin-5-one	<220	<220	
2-Phenyloxazolin-5-one	240	340	9.4
2-Styryloxazolin-5-one	283	378	9.5
4-Methyl-2-phenyloxazolin-5-one	242	352	8.9
4-*p*-Hydroxybenzyl-2-phenyloxazolin-5-one	244	355	

[a] Determined in acetate buffer (pH 5). [b] Determined in 0.1 N NaOH. [c] Determined by spectrophotometric titration at the λ_{max} of the anion.

Experimental Section

Materials. *p*-Nitrophenyl hippurate, *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester, 2-phenyloxazolin-5-one, 4,4-dimethyl-2-phenyloxazolin-5-one, 4,4-dimethyl-2-styryloxazolin-5-one, 4-methyl-2-phenyloxazolin-5-one, oxazolin-5-one, 2-methyloxazolin-5-one, 2-styryloxazolin-5-one, and 4,4-dimethyl-2-*p*-nitrophenyloxazolin-5-one were prepared as described in the previous paper. *N*-Benzoyl-DL-tyrosine was prepared by the method of Fox (1946), and recrystallized from aqueous ethanol: mp 194°, lit. (Fischer, 1899) mp 195–197°. Equimolar amounts of *N*-benzoyl-DL-tyrosine and dicyclohexylcarbodiimide were reacted for 1 hr at room temperature in ethyl acetate. After the mixture was cooled to 0°, it was filtered to remove dicyclohexylurea, and the ethyl acetate was removed under reduced pressure. The resulting oil solidified on trituration with petroleum ether (bp 60–80°), and the solid, DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one, was recrystallized from chloroform–petroleum ether, mp 101–102°. The infrared spectrum showed typical oxazolinone absorption bands at 1824 (carbonyl) and 1646 cm^{-1} ($>C{=}N{-}$). *N*-*trans*-Cinnamoylimidazole was prepared as described by Schonbaum *et al.* (1961). *p*-Thiocresol (Aldrich) was purified by sublimation *in vacuo*, mp 44°. *N*-Benzoyl-L-arginine ethyl ester and cysteine hydrochloride (Mann Research Laboratories) and acetonitrile (Eastman Spectro Grade) were used without further purification. α-Chymotrypsin (three-times crystallized), trypsin (two-times crystallized), and papain (two-times crystallized) were obtained from Worthington Biochemical Corp. Ox liver carboxylesterase was kindly supplied in a highly purified form by Dr. M. T. C. Runnegar. Buffers were prepared and pH values were measured as described in the previous paper (de Jersey *et al.*, 1969).

Methods. Kinetic measurements were made at 25 ± 0.1° on a Cary 14 recording spectrophotometer as described in the previous paper. The ultraviolet absorption spectra of the oxazolinones at neutral pH and in 0.1 N NaOH and the apparent pK_a's (pK_a') determined spectrophotometrically of the various oxazolinones were measured as described for 2-phenyloxazolin-5-one in the previous paper. All of the substrates except *N*-benzoyl-L-arginine ethyl ester were made up as stock solutions in acetonitrile, *N*-benzoyl-L-arginine ethyl ester being made up in water. The normality of α-chymotrypsin solutions was routinely measured by titration with *N*-*trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961) before and after a series of kinetic runs. The normality of the ox liver carboxylesterase solution was determined by titration with *o*-nitrophenyl dimethylcarbamate (Runnegar *et al.*, 1969). The activities of trypsin and papain solutions were determined in rate assays using *N*-benzoyl-L-arginine ethyl ester as substrate, by following the increase in absorbance at 253 mμ (Schwert and Takenaka, 1955).

Results

Ultraviolet Absorption Spectra and Apparent Dissociation Constants of Oxazolinones. The hydrolysis of oxazolinones may be followed by the decrease in ultraviolet absorbance which accompanies the opening of the oxazolinone ring. When the 2-substituent is an aryl- or other α,β-unsaturated group, Δε for hydrolysis is large. Spectral data and apparent pK_a's, determined for a number of saturated oxazolinones are given in Table I.

Spontaneous Hydrolysis of Oxazolinones. The effect of the 2 substituent on the rate of hydrolysis of 4,4-dimethyloxazolin-5-ones was determined at pH 9.20

TABLE II: Substituent Effects in the Hydrolysis of 4,4-Dimethyloxazolin-5-ones at 25°.

2 Substituent	$10^3 k_{obsd}$[a]	Δε (λ)[b] (mμ)
H	1.26	2,910 (220)
CH_3	1.45	~120 (235)
$C_6C_5CH{=}CH$	2.25	9,840 (300)
C_6H_5	2.9	10,300 (250)
p-$NO_2C_6H_5$	5.9	3,700 (280)

[a] In sec^{-1}; determined in a series of sodium borate buffers (pH 9.20) and extrapolated to zero buffer concentration. [b] Δε for conversion of oxazolinone into acid anion under the experimental conditions at the wavelength, λ.

(Table II). Rate constants for the hydrolysis of 2-phenyloxazolin-5-one, 4-methyl-2-phenyloxazolin-5-one, and 4,4-dimethyl-2-phenyloxazolin-5-one at pH 8.1 were determined to test the effect of substitution in the 4 position of the oxazolinone ring (Table III). Imidazole reacts very rapidly with 2-phenyloxazolin-5-one, to form an unstable intermediate, hippurylimidazole. *p*-Nitrophenyl hippurate reacts with imidazole to give the same intermediate. When the reactions were followed in 0.01 M imidazole-HCl (pH 7.15), k_{obsd} for the loss of 2-phenyloxazolin-5-one was estimated at 0.19 sec^{-1} (determined at 250 mμ), whereas k_{obsd} for the release of *p*-nitrophenol from *p*-nitrophenyl hippurate was found to be 9.3×10^{-3} sec^{-1}.

Enzymatic Hydrolysis of 4,4-Dimethyl-2-phenyloxazolin-5-one. The reactions of 4,4-dimethyl-2-phenyloxazolin-5-one with α-chymotrypsin, trypsin, papain, and ox liver carboxylesterase have been investigated. When the reaction of 4,4-dimethyl-2-phenyloxazolin-5-one (4.5×10^{-5} M) with α-chymotrypsin (~1 mg/ml) in 0.1 M phosphate buffer (pH 6.96) is followed at 250 mμ, a rapid decrease in absorbance is followed by a slower zero-order decrease. Extrapolation of the zero-order rate to zero time and comparison with a control experiment using buffer instead of enzyme gave a value for ΔA_{burst} of 0.306. $\Delta\epsilon_{\text{burst}}^{250\,\text{m}\mu}$ was measured by determination of $\Delta\epsilon$ for the hydrolysis of 4,4-dimethyl-2-phenyloxazolin-5-one (−10,310) and $\Delta\epsilon$ for the hydrolysis of the corresponding acyl-enzyme, formed by adding an aliquot of 4,4-dimethyl-2-phenyloxazolin-5-one to an excess of α-chymotrypsin (−880). Therefore, $\Delta\epsilon_{\text{burst}}^{250\,\text{m}\mu} = -9430 \pm 110$. Assuming that $\Delta A/\Delta\epsilon$ is a measure of enzyme concentration, $[\text{E}]_0 = 3.3_0 \times 10^{-5}$ N. Titration of the same enzyme solution with *N-trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961) at 335 mμ gave a value of $3.3_6 \times 10^{-5}$ N for $[\text{E}]_0$. Therefore, the two methods of titration give identical values for enzyme concentration within experimental error. The deacylation rate constant, k_{+3}, for the reaction of 4,4-dimethyl-2-phenyloxazolin-5-one with α-chymotrypsin could not be determined by conventional methods ($[\text{S}]_0 \gg [\text{E}]_0$) because of the relatively rapid spontaneous hydrolysis of 4,4-dimethyl-2-phenyloxazolin-5-one. However, k_{+3} at pH 6.96 was determined by two methods: (i) observation of the entire time course of the reaction with $[\text{E}]_0 > [\text{S}]_0$ at 250 mμ; (ii) use of *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester as an assay substrate. With $[\text{E}]_0 = 6 \times 10^{-5}$ N and $[\text{S}]_0 = 4.5 \times 10^{-5}$ M, an initial large decrease in absorbance at 250 mμ (corresponding to the acylation reaction) was followed by a much slower, smaller, first-order decrease, for which k_{obsd} was estimated at $1.1_0 \times 10^{-3}$ sec^{-1}. To confirm that this small absorbance change (~0.04 absorbance unit) represents deacylation, the reaction was repeated, and aliquots of the reaction mixture, taken at various times after the addition of 4,4-dimethyl-2-phenyloxazolin-5-one, were assayed against *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester at pH 7. Assuming that the observed rate of hydrolysis of *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester was proportional to the concentration of free enzyme, a first-order rate constant of $1.0_9 \times 10^{-3}$ sec^{-1} was obtained. 4,4-Dimethyl-2-phenyloxazolin-5-one reacts readily with trypsin but at a slower rate than with α-chymotrypsin. Whereas acylation of α-chymotrypsin by 4,4-dimethyl-2-phenyloxazolin-5-one at pH 7 is complete in 30 sec, the reaction with trypsin under similar conditions takes about 10 min. Spon-

TABLE III: Substituent Effects in the Hydrolysis of 2-Phenyloxazolin-5-ones at 25°.

Oxazolinone	$10^3 k_{\text{obsd}}$[a]	$\Delta\epsilon$ at 250 mμ
2-Phenyloxazolin-5-one	1.7	7,650
4-Methyl-2-phenyloxazolin-5-one	2.8	~10,000[b]
4,4-Dimethyl-2-phenyloxazolin-5-one	0.3_2	10,300

[a] Determined in a series of Tris-HCl buffers at pH 8.10, and extrapolated to zero Tris concentration; k_{obsd} (sec^{-1}) determined at 250 mμ. [b] See de Jersey *et al.* (1969).

TABLE IV: Kinetic Constants for the Enzyme-Catalyzed Hydrolyses of 2-Phenyloxazolin-5-one and *p*-Nitrophenyl Hippurate at 25°.

		k_{cat} (sec^{-1})		K_{m} (mM)	
Enzyme	pH	2-Phenyloxazolin-5-one	*p*-Nitrophenyl Hippurate	2-Phenyloxazolin-5-one	*p*-Nitrophenyl Hippurate
α-Chymotrypsin	6.96[a]	0.53	0.40 0.52[d]	0.008	0.02
	5.08[b]	0.011	0.010[e]		0.009[e]
Ox liver carboxylesterase	6.18[c]	134	12	0.074	1.3

[a] 0.1 M phosphate buffer; 3% (v/v) acetonitrile in reaction mixture. [b] 0.1 M acetate buffer; 3% acetonitrile. [c] 0.09 M phosphate buffer; 12.7% acetonitrile. [d] Calculated using points at low substrate concentration. [e] R. L. Blakeley, unpublished observations in this laboratory.

taneous hydrolysis during this time makes the extrapolation of the pseudo-steady-state rate to zero time less accurate. However, the size of the burst could be determined to within 5% or better, indicating that 4,4-dimethyl-2-phenyloxazolin-5-one could be used to titrate trypsin as well as α-chymotrypsin.

Papain (1.4 mg/ml) in 0.05 M phosphate buffer (pH 6.06) containing 5×10^{-4} M EDTA was activated by 2.5×10^{-4} M cysteine. When activation was complete (as adjudged by rate assay with *N*-benzoyl-L-arginine ethyl ester as substrate), an aliquot of 4,4-dimethyl-2-phenyloxazolin-5-one was added to 3 ml of papain and the reaction observed at 250 mμ. With $[\text{4,4-dimethyl-2-phenyloxazolin-5-one}]_0 = 9.08 \times 10^{-5}$ M, a burst of 0.27 absorbance unit was observed. With $[\text{4,4-dimethyl-2-phenyloxazolin-5-one}]_0 = 1.35 \times 10^{-4}$ M, aliquots of the reaction mixture were taken at various times after the addition of 4,4-dimethyl-2-phenyloxazolin-5-one, and assayed against *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester at pH 7. A steady state was reached in less than 10 min, in which ~8% of the enzyme remained in the free state, indicating that $[P_1]_{\text{burst}} \sim 0.92[E]_0$. Activator-free papain was prepared by activation with *p*-thiocresol in toluene (Soejima and Shimura, 1961), and by activation with 5×10^{-3} M cysteine followed by Sephadex G-25 chromatography. Using papain prepared by either of these procedures, the burst at 250 mμ could be observed as before.

4,4-Dimethyl-2-phenyloxazolin-5-one proved to be a good substrate for ox liver carboxylesterase. Kinetic constants for the hydrolysis in 0.1 M phosphate buffer (pH 6.96) at 25° were evaluated from data for a Lineweaver–Burk plot by the method of least squares: $k_{\text{cat}} = 24.7\ \text{sec}^{-1}$; $K_m = 3.2 \times 10^{-5}$ M.

2-Phenyloxazolin-5-one and p-Nitrophenyl Hippurate as Substrates for α-Chymotrypsin and Ox Liver Carboxylesterase. Kinetic constants for the hydrolysis of 2-phenyloxazolin-5-one and *p*-nitrophenyl hippurate by α-chymotrypsin were determined at pH 6.97 and 5.08. The hydrolysis by ox liver carboxylesterase was studied at pH 6.18. Results obtained are collected in Table IV. All of the kinetic constants, except those for the α-chymotrypsin-catalyzed hydrolysis of 2-phenyloxazolin-5-one at pH 5.08, were evaluated from data for Lineweaver–Burk plots by the method of least squares. For 2-phenyloxazolin-5-one with α-chymotrypsin at pH 5.08, this method was not possible because of the rapid spontaneous hydrolysis. However, it was possible to observe the deacylation reaction directly at 250 mμ with enzyme concentration higher than substrate concentration. The significance of the measured kinetic constants for the α-chymotrypsin-catalyzed hydrolyses in terms of the acyl-enzyme mechanism (eq 1) was determined by

$$E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_{+2}} ES' + P_1 \xrightarrow{k_{+3}} E + P_2 \qquad (1)$$

studying the reactions at pH 5 at higher enzyme concentrations. With *p*-nitrophenyl hippurate, a burst of *p*-nitrophenol was observed at 317 mμ. Ouellet and Stewart (1959) derived the equation relating the size of the burst to the enzyme concentration (eq 2). By

$$[P_1]_{\text{burst}} = \left[\frac{k_{+2}}{k_{+2} + k_{+3}} \frac{[S]_0}{[S]_0 + K_m}\right]^2 [E]_0 \qquad (2)$$

substitution of the known values of $[S]_0$, $[E]_0$, K_m, and $[P_1]_{\text{burst}}$ in the equation, the ratio $k_{+2}/(k_{+2} + k_{+3})$ is obtained. For 2-phenyloxazolin-5-one, there is no P_1. However, when the reaction of 2-phenyloxazolin-5-one with α-chymotrypsin is observed at pH 5 (250 mμ), an initial very rapid decrease in absorbance is observed. The concentration of oxazolinone corresponding to this rapid decrease is equated to $[P_1]_{\text{burst}}$. For both 2-phenyloxazolin-5-one and *p*-nitrophenyl hippurate, results of the burst experiments establish that $k_{+2} \gg k_{+3}$. The ox liver carboxylesterase catalyzed hydrolyses were carried out in 12.7% (v/v) acetonitrile buffer, to permit use of higher concentrations of *p*-nitrophenyl hippurate.

TABLE V: Effect of 2-Phenyloxazolin-5-one on the Initial Rate of Hydrolysis of *p*-Nitrophenyl Hippurate by α-Chymotrypsin.[a]

[2-Phenyloxazolin-5-one]$_0$ (μM)	Initial Rate[b] (μM sec^{-1})	Initial Rate/Rate in Absence of 2-Phenyloxazolin-5-one
	0.715	1.00
14.7	0.560	0.78
29.4	0.460	0.64
51.4	0.355	0.50
73.4	0.287	0.40

[a] 25°; $[p\text{-nitrophenyl hippurate}]_0 = 8.97 \times 10^{-5}$ M; $[E]_0 = 1.59 \times 10^{-6}$ N; aliquots of 2-phenyloxazolin-5-one and *p*-nitrophenyl hippurate in acetonitrile (100 μl total) were added simultaneously to 3.1 ml of a solution of α-chymotrypsin in 0.1 M phosphate buffer (pH 7.00), and the release of *p*-nitrophenol observed at 400 mμ. [b] Calculated using $\epsilon_{400\text{m}\mu}$ for *p*-nitrophenol = 8180 under the experimental conditions.

Effect of 2-Phenyloxazolin-5-one on the Hydrolysis of p-Nitrophenyl Hippurate by α-Chymotrypsin. The effect of added 2-phenyloxazolin-5-one on the turnover of *p*-nitrophenyl hippurate by α-chymotrypsin at pH 7 was determined (Table V). In a similar experiment ($[E]_0 = 1.6 \times 10^{-6}$ N, $[p\text{-nitrophenyl hippurate}]_0 = 9.1 \times 10^{-5}$ M), the turnover of *p*-nitrophenyl hippurate by α-chymotrypsin in 0.025 M phosphate buffer (pH 7.56) was measured by following the release of *p*-nitrophenol at 400 mμ. Enzyme was added before and at various times after the addition of *p*-nitrophenyl hippurate. Addition of *p*-nitrophenyl hippurate 2 min before α-chymotrypsin gave an initial rate which was only 74% of that obtained when the enzyme was added first. Further, direct observation of the acylation of α-chymotrypsin at pH 5 by 2-phenyloxazolin-5-one and *p*-nitrophenyl hippurate (each at a concentration of 10^{-4} M) indicates that 2-phenyloxazolin-5-one acylates the enzyme faster than does *p*-nitrophenyl hippurate under these conditions.

TABLE VI: The Hydrolysis of DL-4-*p*-Hydroxybenzyl-2-phenyloxazolin-5-one by α-Chymotrypsin at pH 4. Effect of Enzyme Concentration.[a]

$10^6[E]_0$[b] (N)	ΔA_{burst}	Equiv[c] [DL-4-(*p*-Hydroxybenzyl)-2-phenyloxazolin-5-one] (μM)	[DL-4-(*p*-Hydroxybenzyl)-2-phenyloxazolin-5-one]$_{\text{hydrolyzed}}$ (μM)	[DL-4-(*p*-Hydroxybenzyl)-2-phenyloxazolin-5-one]$_{\text{hydrolyzed}}$[e]/[DL-4-(*p*-Hydroxybenzyl)-2-phenyloxazolin-5-one]$_0$	[DL-4-(*p*-Hydroxybenzyl)-2-phenyloxazolin-5-one]$_{\text{hydrolyzed}}$[d]/[E]$_0$
21.6	0.717	90.0	68.4	0.51	3.2
10.8	0.563	70.6	59.8	0.44	5.5
5.4	0.399	50.1	44.7	0.33	8.3
2.7	0.260	32.6	29.9	0.22	11.1
1.35	0.152	19.1	17.7	0.13	13.1

[a] Reaction observed at 250 mμ and 25° in 0.05 M acetate buffer (pH 4.02). [b] Determined by titration with *N-trans*-cinnamoylimidazole. [c] $\Delta\epsilon$ at 250 mμ for hydrolysis of DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one is 7970. [d] [DL-4-(*p*-Hydroxybenzyl)-2-phenyloxazolin-5-one]$_{\text{hydrolyzed}}$ = equivalent [DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one] − $[E]_0$. [e] [DL-4-(*p*-Hydroxybenzyl)-2-phenyloxazolin-5-one]$_0$ = 1.35×10^{-4} M.

The Reaction of Racemic DL-*4-(p-Hydroxybenzyl)-2-phenyloxazolin-5-one with α-Chymotrypsin.* When an aliquot of DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one was added to a solution of α-chymotrypsin at pH 4, and the reaction was observed at 250 mμ, an initial rapid decrease in absorbance (or burst) was followed by a much slower decrease in absorbance corresponding to the spontaneous hydrolysis of DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one. It was postulated that the apparent inhibition of enzyme activity was due to the formation of benzoyl-D-tyrosyl-α-chymotrypsin, which should be stable at pH 4 (eq 3). The effect of enzyme concentration on the size of the burst was determined at a fixed concentration of DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one (Table VI). The concentration of DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one converted into product acid ([DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one]$_{\text{hydrolyzed}}$) was calculated assuming that the α-chymotrypsin was converted stoichiometrically into acyl-enzyme. Figure 1 shows a plot of $[E]_0$/[DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one]$_{\text{hydrolyzed}}$ against $[E]_0$/[DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one]$_0$ (data from Table VI), giving a limiting value of 17 for the ratio [DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one]$_{\text{hydrolyzed}}/[E]_0$.

$$E + \text{L-PHBO[1]} \overset{K_S^{L}}{\rightleftharpoons} E\cdot\text{L-PHBO} \xrightarrow{K_{+2}^{L}} \text{L-acyl-E} \xrightarrow{K_{+3}^{L}} \text{L-acid} + E$$

$$E + \text{D-PHBO} \overset{K_S^{D}}{\rightleftharpoons} E\cdot\text{D-PHBO} \xrightarrow{K_{+2}^{D}} \text{D-acyl-E} \qquad (3)$$

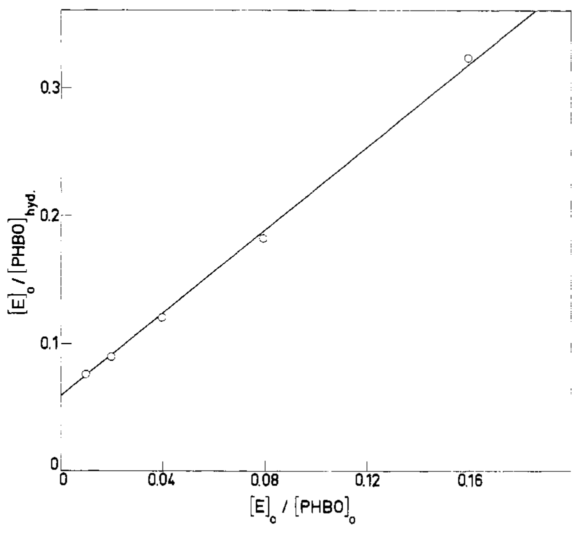

FIGURE 1: Hydrolysis of DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one by α-chymotrypsin at pH 4. Plot of $[E]_0/[\text{PhBO}]_{\text{hydrolyzed}}$ *vs.* $[E]_0/[\text{PHBO}]_0$ (see text; data from Table VI).

When the hydrolysis of DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one by α-chymotrypsin was followed in 0.1 M phosphate buffer (pH 7.05), a rapid burst was followed by slower turnover. By choosing an enzyme concentration at which all of the L isomer was hydrolyzed instantly, the turnover of the D-acyl-enzyme could be observed. With $[E]_0 = 1.11 \times 10^{-5}$ N and [DL-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one]$_0$ varied from 4.6 to 18.2×10^{-5} M, a linear Lineweaver–Burk plot was obtained, giving values of k_{cat} (0.075 sec^{-1}) and K_m (8.0×10^{-6} M). The reaction mixtures contained 2.5% (v/v) acetonitrile.

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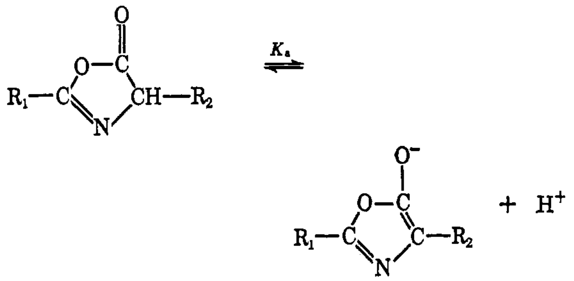
[1] PHBO = 4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one.

Discussion

Racemization of Oxazolinones. The apparent pK_a values given in Table I refer to the ionization (de Jersey *et al.*, 1969)



For many years, it has been supposed that the base-catalyzed enolization of oxazolinones is responsible for their facile racemization (Bergmann and Zervas, 1928). Karrer and Keller (1943) observed a transient blue color on the addition of oxazolinones with a highly conjugated system in the 2 position to alkali, and attributed the color to the formation of the unstable enolate ion. The pK_a' values thus give a quantitative measure of the acidity of the hydrogen(s) in the 4 position of the oxazolinones. Similarly, they furnish a ready explanation for the ease of racemization of oxazolinones. The effect of the 2 substituent on the apparent pK_a is small, but substitution at the 4 position has a considerable effect on the pK_a' (Table I).

Spontaneous Hydrolysis of Saturated Oxazolinones. Previous fragmentary results indicate that saturated oxazolinones are very unstable in aqueous solution, especially at alkaline pH (Cornforth, 1949; Goodman and Levine, 1964). The substituent in the 2 position has a considerable effect on the rate of hydrolysis of 4,4-dimethyloxazolin-5-ones (Table II) and 4-unsubstituted oxazolin-5-ones (de Jersey *et al.*, 1969). A Bronsted plot of the data in Table II is linear, with the exception of the point corresponding to 4,4-dimethyloxazolin-5-one. The pH–rate profile for the hydrolysis of 2-phenyloxazolin-5-one has already been discussed (de Jersey *et al.*, 1969). A single methyl substitution in the 4 position increases the rate of hydrolysis, while 4,4-dimethyl substitution produces a ten fold decrease in k_{obsd}, measured at pH 8.10 (Table III). This decrease is presumably due to steric hindrance to attack at the carbonyl group (Zerner and Bender, 1961).

Comparison of the lability of oxazolinones with that of other activated derivatives of *N*-acylamino acids in aqueous solution is difficult, since such activated derivatives hydrolyze through oxazolinone intermediates. The rate constant for the hydrolysis of *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester at pH 8.1, estimated from the apparent alkaline rate constant of 156 M^{-1} sec^{-1}, is 2.0×10^{-4} sec^{-1}, compared with 1.7×10^{-3} sec^{-1} determined for 2-phenyloxazolin-5-one at pH 8.1. Both 2-phenyloxazolin-5-one and *p*-nitrophenyl hippurate react with imidazole to form hippurylimidazole. k_{obsd} for the reaction of 2-phenyloxazolin-5-one in 0.01 M imidazole-HCl buffer (pH 7.15) is approximately 20 times greater than k_{obsd} for the reaction of *p*-nitrophenyl hippurate under the same conditions. This factor of 20 would include contributions from differences in steric hindrance to nucleophilic attack at the carbonyl group and differences in inherent reactivity of oxazolinones and *p*-nitrophenyl esters toward nucleophiles. In this connection, Avison (1955) has used the facile reaction of saturated oxazolinones with orthophosphate in aqueous pyridine to prepare acyl phosphates in quantitative yield.

Enzymatic Hydrolysis of 4,4-Dimethyl-2-phenyloxazolin-5-one. The reactions of 4,4-dimethyl-2-phenyloxazolin-5-one with α-chymotrypsin, trypsin, and papain are characterized by a rapid acylation reaction (the burst) followed by a slower deacylation reaction. The size of the burst could be measured by observation of the reactions at 250 mμ. For α-chymotrypsin, the agreement between the values of $[\mathrm{E}]_0$ determined by titration with 4,4-dimethyl-2-phenyloxazolin-5-one and with *N*-*trans*-cinnamoylimidazole indicates immediately that in the α-chymotrypsin-catalyzed hydrolysis of 4,4-dimethyl-2-phenyloxazolin-5-one, $k_{+2} \gg k_{+3}$ and $[\mathrm{S}]_0 \gg K_{\mathrm{m}}([\mathrm{S}]_0 = 4.5 \times 10^{-5}\ \mathrm{M})$ (eq 2). The agreement also indicates that under the conditions used, 4,4-dimethyl-2-phenyloxazolin-5-one reacts exclusively at the active site (*cf.* Baranowski *et al.*, 1963). Similarly, the reaction of 4,4-dimethyl-2-phenyloxazolin-5-one with papain was followed directly by observing the burst at 250 mμ, or indirectly by estimation of the residual free enzyme using *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester as assay substrate. The agreement between the two procedures indicates that the observed burst represents acylation of the active site. Compared with other reagents which have been used as titrants for hydrolytic enzymes (*e.g.*, *N*-*trans*-cinnamoylimidazole), 4,4-dimethyl-2-phenyloxazolin-5-one is relatively nonspecific and could be developed as a titrant for papain and trypsin as well as for α-chymotrypsin. 4,4-Dimethyl-2-phenyloxazolin-5-one is also unusual in that although it is a derivative of an *N*-acylamino acid, the acyl-enzymes formed are sufficiently stable to allow titration of the enzymes at neutral pH. However, 4,4-dimethyl-2-phenyloxazolin-5-one has at least two disadvantages as a titrant. Firstly, the relatively fast spontaneous hydrolysis limits the accuracy with which the size of the burst can be measured. Secondly, protein solutions of the concentration required for titration absorb quite strongly at 250 mμ. Use of the more highly conjugated 4,4-dimethyl-2-styryloxazolin-5-one (Δε ~ 10,000 at 300 mμ) could possibly overcome this difficulty. However, specificity becomes increasingly important, since preliminary experiments with papain indicate that acylation by 4,4-dimethyl-2-styryloxazolin-5-one is considerably slower than that by 4,4-dimethyl-2-phenyloxazolin-5-one. 4,4-Dimethyl-2-phenyloxazolin-5-one proved to be too good a substrate for ox liver carboxylesterase for observation of a burst to be feasible. When 4,4-dimethyl-2-phenyloxazolin-5-one or 2-phenyloxazolin-5-one is added to an excess of α-chymotrypsin, the deacylation reaction can be followed directly at 250 mμ. Δε for the deacylation of benzoylaminoisobutyryl-α-chymotrypsin at 250 mμ is ~880. Assays with *N*-benzyl-

TABLE VII: Catalytic Rate Constants for α-Chymotrypsin- and Ox Liver Carboxylesterase-Catalyzed Hydrolyses at 25°.

Substrate	α-Chymotrypsin [a] k_{cat} (sec^{-1})	Ox Liver Carboxylesterase k_{cat} (sec^{-1})	$\frac{k_{cat}\text{ Carboxylesterase}}{k_{cat}\ \alpha\text{-Chymotrypsin}}$
2-Phenyloxazolin-5-one	0.53	134[b]	2.5×10^2
p-Nitrophenyl butyrate	3×10^{-3} [c]	110[d]	3.7×10^4
4,4-Dimethyl-2-phenyloxazolin-5-one	1.1×10^{-3}	25[e]	2.3×10^4
p-Nitrophenyl trimethylacetate	9×10^{-5} [f]	5.3[d]	5.9×10^4

[a] k_{cat} at pH 7.0. [b] pH 6.18; 12.7% CH_3CN. [c] Fife and Milstein (1967). [d] pH 7.45; 13% CH_3CN; Runnegar (1968). [e] pH 6.96; 3% CH_3CN. [f] Bender *et al.* (1962).

oxycarbonylglycine *p*-nitrophenyl ester indicated that the small absorbance change observed does indeed represent deacylation.

Comparison of 2-Phenyloxazolin-5-one and p-Nitrophenyl Hippurate as Substrates for Hydrolytic Enzymes. The significance of the experimentally determined rate constants, k_{cat} and K_m, for a particular substrate in terms of k_{+2}, k_{+3}, and K_S (eq 1) is uncertain in the absence of other evidence (Zerner and Bender, 1964). Values of k_{cat} for the α-chymotrypsin-catalyzed hydrolysis of 2-phenyloxazolin-5-one and *p*-nitrophenyl hippurate are identical at pH 5 and at 7 (Table IV). Burst experiments at pH 5 indicate that for both substrates, $k_{+2} \gg k_{+3}$. The three-step mechanism therefore requires that the k_{cat} values be identical. With ox liver carboxylesterase at pH 6.18, k_{cat} for 2-phenyloxazolin-5-one is greater than k_{cat} for *p*-nitrophenyl hippurate by a factor of 11, and K_m for 2-phenyloxazolin-5-one is lower than K_m for *p*-nitrophenyl hippurate by a similar factor. Evidence for the mechanism of eq 1 in the action of liver carboxylesterases has recently been obtained (Stoops *et al.*, 1969). Since k_{+3} must be the same for 2-phenyloxazolin-5-one and *p*-nitrophenyl hippurate, the differences in k_{cat} values must reflect differences in the acylation rate constants. The k_{cat} obtained for 2-phenyloxazolin-5-one (134 sec^{-1}) reveals that it is a very good substrate for ox liver carboxylesterase, and it is likely the k_{+3} would be at least largely rate limiting. Therefore, the presence of the *p*-nitrophenyl group has a large effect on k_{+2}. Other experiments have indicated that the *p*-nitro group is the site of considerable steric hindrance in the acylation reaction, and an explanation of this steric hindrance has been proposed (Augusteyn *et al.*, 1969). 2-Phenyloxazolin-5-one is thus a much more satisfactory activated derivative of hippuric acid than *p*-nitrophenyl hippurate for the determination of the deacylation rate constant for hippuryl carboxylesterase (ox liver).

Relative Catalytic Efficiencies of α-Chymotrypsin and Ox Liver Carboxylesterase. The data in Table VII allow many specificity comparisons to be made. With all of the substrates listed, the carboxylesterase is much more efficient than α-chymotrypsin. The factor is least with 2-phenyloxazolin-5-one, showing that the specificity of α-chymotrypsin is starting to be seen. Comparison of the relative k_{cat}'s for 2-phenyloxazolin-5-one and 4,4-dimethyl-2-phenyloxazolin-5-one shows that the effect of the *gem*-dimethyl substitution is much more marked in α-chymotrypsin-catalyzed hydrolysis than in ox liver carboxylesterase-catalyzed hydrolysis.

The Accuracy of Kinetic Constants of N-Acylamino Acid Esters. Three experiments indicate that 2-phenyloxazolin-5-one acylates α-chymotrypsin more readily than does *p*-nitrophenyl hippurate: direct observation of the acylation reaction at pH 5; the competition experiment reported in Table VII; and the relative K_m values at pH 7 (Table IV). Several ways, therefore, in which kinetic constants for activated *N*-acylamino acid esters could be rendered inaccurate may be envisaged: (i) if the substrate is added before the enzyme to a buffer in which the spontaneous hydrolysis is appreciable (*e.g.*, the experiment with *p*-nitrophenyl hippurate and α-chymotrypsin at pH 7.56); (ii) if the substrate is unstable in the organic solvent used for making up the stock solutions (especially if the solvent is slightly basic), an appreciable amount of oxazolinone could build up in the stock solution; (iii) if the spontaneous rate is significant compared with the enzymatic rate (Table IV).

Finally, errors caused by the presence of oxazolinone are likely to be most significant if the acylation reaction is being studied.

Reaction of α-Chymotrypsin with DL*-4-(p-Hydroxybenzyl)-2-phenyloxazolin-5-one, the Oxazolinone of a Specific Substrate.* From the kinetic scheme postulated to explain the observations at pH 4 (eq 3), the following relationship may be derived

$$\left[\frac{[\mathrm{PHBO}]_{\mathrm{hydrolyzed}}}{[\mathrm{E}]_0}\right]_{\mathrm{lim}} \simeq \frac{k_{+2}{}^{\mathrm{L}}}{k_{+2}{}^{\mathrm{D}}}\,\frac{K_{\mathrm{S}}{}^{\mathrm{D}}}{K_{\mathrm{S}}{}^{\mathrm{L}}}$$

The derivation assumes that in the limiting case (Figure 1), the concentrations of both L- and D-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one do not change during the reaction. The value of 17 obtained for $[[\mathrm{PHBO}]_{\mathrm{hydrolyzed}}/[\mathrm{E}]_0]_{\mathrm{lim}}$ is therefore a measure of the extent to which α-chymotrypsin favors the L isomer in the acylation reaction. The value of k_{cat} in the reaction of α-chymotrypsin with D-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one (0.075 sec^{-1} at pH 7.05) may be compared with the k_{cat} determined for the α-chymotrypsin-catalyzed hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (~100 sec^{-1} at pH 7) (Gutfreund and Hammond, 1959). The k_{cat} for D-4-(*p*-hydroxybenzyl)-2-phenyloxazolin-5-one reflects k_{+3}, since at pH 4, all of the enzyme is eventually converted into stable D-acyl-enzyme. k_{cat} for benzoyl-L-tyrosine ethyl ester certainly reflects k_{+3} also. Therefore, the specificity factor in the deacylation reaction is approximately 1000, in favor of the L isomer.

If the present results may be extrapolated to other substrates, they suggest that the specificity favoring the L isomer of an activated "specific" substrate is associated more with the deacylation reaction than with the acylation reaction (*cf.* Bender *et al.*, 1964).

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References

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

Augusteyn, R. C., de Jersey, J., Webb, E. C., and Zerner, B. (1969), *Biochim. Biophys. Acta 171*, 128.

Avison, A. W. D. (1955), *J. Chem. Soc.*, 732.

Baranowski, T., Kochman, M., Nowak, K., and Siemion, I. (1963), *Bull. Acad. Pol. Sci. Ser. Sci. Biol. 11*, 107.

Bender, M. L., Kézdy, F. J., and Gunter, C. R. (1964), *J. Amer. Chem. Soc. 86*, 3714.

Bender, M. L., Schonbaum, G. R., and Zerner, B. (1962), *J. Amer. Chem. Soc. 84*, 2562.

Bergmann, M., Stern, F., and Witte, C. (1926), *Justus Liebigs Ann. Chem. 449*, 277.

Bergmann, M., and Zervas, L. (1928), *Biochem. Z. 203*, 280.

Carter, H. E. (1946), *Org. Reactions 3*, 198.

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

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., Willadsen, P., and Zerner, B. (1969), *Biochemistry 8*, 1959 (this issue, paper 1).

Fife, T. H., and Milstein, J. B. (1967), *Biochemistry 6*, 2901.

Fischer, E. (1899), *Chem. Ber. 32*, 3638.

Fox, S. W. (1946), *J. Amer. Chem. Soc. 68*, 194.

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

Goodman, M., and McGahren, W. J. (1967), *Tetrahedron 23*, 2017, 2031.

Gutfreund, H., and Hammond, B. R. (1959), *Biochem. J. 73*, 526.

Karrer, P., and Keller, R. (1943), *Helv. Chim. Acta 26*, 50.

Leplawy, M. T., Jones, D. S., Kenner, G. W., and Sheppard, R. C. (1960), *Tetrahedron 11*, 39.

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

Ouellet, L., and Stewart, J. A. (1959), *Can. J. Chem. 37*, 737.

Runnegar, M. T. C. (1968), Ph.D. Thesis, University of Queensland, Brisbane, Australia.

Runnegar, M. T. C., Webb, E. C., and Zerner, B. (1969), *Biochemistry 8*, 2018 (this issue; paper 9).

Schonbaum, G. R., Zerner, B., and Bender, M. L. (1961), *J. Biol. Chem. 236*, 2930.

Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.

Soejima, M., and Shimura, K. (1961), *J. Biochem. (Tokyo) 49*, 260.

Stoops, J. K., Horgan, D. J., Runnegar, M. T. C., de Jersey, J., Webb, E. C., and Zerner, B. (1969), *Biochemistry 8*, 2026 (this issue; paper 10).

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.

Zerner, B., and Bender, M. L. (1964), *J. Amer. Chem. Soc. 86*, 3669.