warm and very thick. It was placed in an oil bath, heated at 90°, and 12 g. (0.11 mole) of trimethylchlorosilane were slowly added with mechanical stirring. The mixture was filtered after 2 hr. at 90°, the filtrate was concentrated on a rotating evaporator at 60°. The product crystallized in large colorless crystals on cooling to room temperature after most of the solvent was removed; after two recrystallizations from dry hexane, m.p. 53–56°. *Anal.* Calcd.: C, 69.8; H, 9.1; N, 5.1. Found: C, 70.0; H, 9.4; N, 5.0.

11. N,N-Dimethyl-N′-phenyl-N′-trimethylsilylthiourea.—Phenyl isothiocyanate (6.75 g., 0.05 mole) in 20 ml. of anhydrous ether was added dropwise over a 15-min. period to a magnetically stirred solution of 5.85 g. (0.05 mole) of N-trimethylsilyl-N,N-dimethylamine in 20 ml. of anhydrous ether. After stirring under a blanket of dry nitrogen for 7 hr., the ether was pulled off at room temperature, leaving a crystalline white solid which was recrystallized from *n*-hexane giving white needles melting at 52–53°

[CONTRIBUTION FROM THE INSTITUTE OF MOLECULAR BIOLOGY AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF OREGON, EUGENE, OREGON]

The Reaction of Formohydroxamic Acid with Acyl Derivatives in Neutral Aqueous Solution

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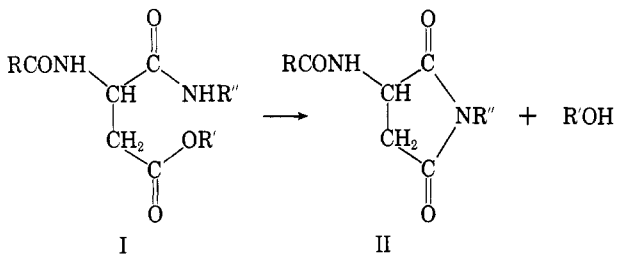
A peculiar reaction of a β-alkyl ester of an aspartylserine peptide with neutral hydroxylamine, yielding formohydroxamic acid (FHA) in nearly stoichiometric quantities, has been observed. The reaction of FHA with acylating agents has been studied. Acylation occurs readily with formohydroxamate anion, the rate of acylation being unusually rapid for an anionic nucleophile with this pK_a (8.70). The acylation of hydroxamic acids by acylimidazoles involves the complementary charged species acylimidazolium$^{\ominus}$ + hydroxamate$^{\ominus}$, to the apparent exclusion of other conjugate acid–base species. These latter acylation reactions are extremely rapid; the product of the reaction is the N,O-diacylhydroxylamine. In contrast to other N,O-diacylhydroxylamines, N-formyl-O-acylhydroxylamines deacylate rapidly at neutral pH. This reaction involves elimination of acylate$^{\ominus}$ rather than hydrolysis.

In a previous communication,[2a] it was reported that derivatives of the dipeptide L-aspartyl-L-serine exhibited chemical reactivity which was not anticipated on the basis of the well-investigated reactivities of the aggregate constituent monofunctional side chains. One such noteworthy chemical phenomenon is the ease with which β-esters of aspartyl peptides (I) cyclize to form 5-membered imide rings (II). The imide ring was found to undergo facile (hydroxyl ion catalyzed) hydrolysis to a mixture of the corresponding α- and β-carboxylates.



I II

Another notable reaction of the above-mentioned aspartylseryl peptide is the hydroxylaminolysis of the β-ester at neutral pH. Formation of the N-hydroxamate from the ester in 1 *M* hydroxylamine at pH 6 (50% aqueous dioxane) occurs at a rate far in excess of the rate of formation of the cyclic imide II from the ester in the absence of NH_2OH, under the otherwise similar conditions. From the hydroxylaminolysis reaction mixture (see Experimental) a hydroxamic acid product was isolated which appeared to catalyze the hydrolysis of acyl derivatives in aqueous solution. The purified product was shown to be formohydroxamic acid. The apparent catalytic reactivity of formohydroxamic acid was found to be typical of hydroxamic acids. The formation of acylate anion was found to proceed *via* a two-step "acylation–deacylation" mechanism, characteristic of the hydrolysis of acyl derivatives catalyzed by tertiary bases (and imidazoles) and by proteolytic enzymes.[2b]

Results and Discussion

The utility of cinnamoyl derivatives as models for the study of acylation reactions has been elegantly demonstrated by Bender and co-workers.[3] Cinnamoylimidazole (CI), and other chromophoric acylating agents, have been employed in this study (following, in essence, the method of Bender),[3] in order to characterize any intermediates which might form in a catalyzed acylation reaction. Since in the experiments reported herein the formation of cinnamate from cinnamoylating agents was found to be promoted by formohydroxamic acid *via* a cinnamoyl intermediate, a number of cinnamoylhydroxamate derivatives were prepared, and their ultraviolet spectra recorded. Pertinent data are listed in Table I.

The Reaction of Cinnamoylimidazole with Formohydroxamic Acid (FHA).—The reaction of formohydroxamic acid (FHA) with cinnamoylimidazole (CI) was followed spectrophotometrically. Figure 1 shows a typical run at 25°, pH 6.86 (0.05 *M* phosphate buffer). Under these conditions (excess FHA) there is a rapid disappearance of CI with a concomitant increase of a cinnamoyl intermediate with an ultraviolet absorbance maximum (λ_{max}) at 282 mμ. This intermediate subsequently undergoes deacylation at a relatively slower rate to yield cinnamate ion (λ_{max} 269.5 mμ). The rate of the first step (acylation) is first order in FHA concentration, whereas the second step (deacylation) is independent of the concentration of free FHA. Both reactions were studied as a function of pH. The dependencies of the first-order rate constants on pH are illustrated in Fig. 2. Both acylation and deacylation steps are optimally rapid near neutrality,

(1) On leave of absence from the Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel.

(2) (a) S. A. Bernhard, A. Berger, J. H. Carter, E. Katchalski, M. Sela, and Y. Shalitin, *J. Am. Chem. Soc.*, **84**, 2421 (1962); (b) M. L. Bender, *ibid.*, **84**, 2582 (1962).

(3) M. L. Bender, G. R. Schonbaum, and B. Zerner, *ibid.*, **84**, 2540 (1962).

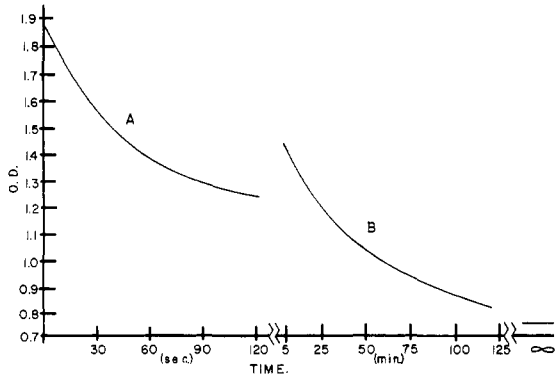
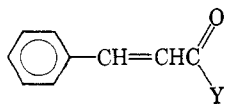


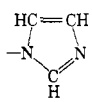
Fig. 1.—Acylation and deacylation of formohydroxamic acid with cinnamoylimidazole at 25° in phosphate buffer (0.05 M), pH 6.86, 0.67% CH_3CN; [FHA] = 2.33×10^{-3} M, [CI] = 1.00×10^{-4} M: curve A, at 320 mμ; curve B, at 290 mμ.

acylation decreasing at higher pH and deacylation decreasing at lower pH. When the reactions were run at concentrations of FHA of ~$1\text{–}2 \times 10^{-3}$ M, acylation was essentially complete before any appreciable de-

TABLE I

SPECTRAL CHARACTERISTICS OF CINNAMOYLHYDROXAMATES AND OTHER REFERENCE COMPOUNDS



Y =	λ_{max},[a] mμ	ε × 10^{-4}[a] (O.D., M^{-1} cm.$^{-1}$)
	307	2.5
—NHOH	274	2.4
—NH—O—C(=O)—CH_3[b]	274	2.9[c]
—O—NH—C(=O)—CH_3[b]	286	2.3[c]
—ONH_2[d]	280	2.2
—$O^{\ominus}$	269.5	2.0
—OCH_3	279	2.2
—OH	280	2.2
—NH_2	272	2.1
—$OCH_2CH<^{NHCOCH_3}_{CONH_2}$[e]	282	2.2

[a] In 0.05 M phosphate buffer, pH 6.87, 0.8% acetonitrile. [b] The structures of these two diacylhydroxylamines have not been investigated in detail; both were prepared by the reaction of the mono-N-hydroxamate with the corresponding acylimidazole. [c] In acetate buffer, pH 4.0. The spectrum is pH dependent (see text). [d] This compound has not been isolated. The structure has been inferred by spectral identification of a metastable intermediate in the reaction of N-propionyl-O-cinnamoylhydroxylamine in 0.06 M NH_2OH, pH 6.0, and the report of Jencks (ref. 5) that acyl-O-hydroxylamines are metastable intermediates in such reactions. [e] Reference 3.

acylation or uncatalyzed hydrolysis of cinnamoylimidazole had occurred. In this way, the ultraviolet absorption spectra of the acyl intermediates could be determined during the course of the kinetic experiments described above. The ultraviolet spectrum of the cinnamoyl intermediate shifts to higher wave lengths with decreasing pH, as is shown in Fig. 3. At both 290 and 270 mμ the difference in extinction between the high pH (ϵ_B) and low pH (ϵ_A) cinnamoyl intermediates are large. On the assumption that the two extreme spectra, at low and at high pH, represent the acidic

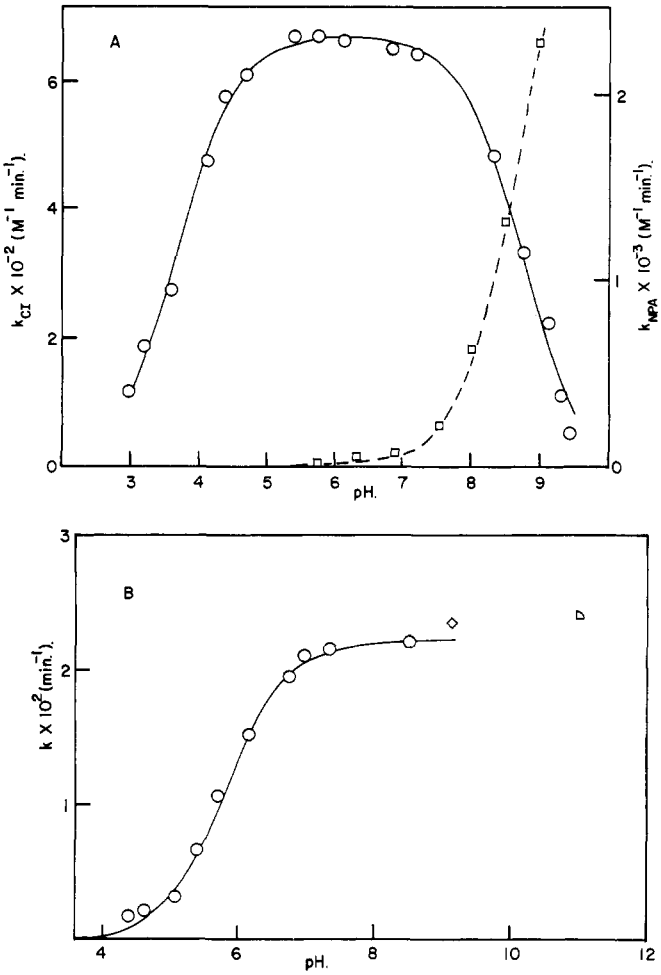


Fig. 2.—Dependence of specific rate of reaction on pH at 25° and μ = 0.10. A. Acylation reactions: ———, acylation of FHA with cinnamoylimidazole, calculated for pK_{CIH^+} = 3.66 and pK_{FHA} = 8.70; ·····, acylation of FHA with p-nitrophenyl acetate, calculated for pK_{FHA} = 8.70. B. Deacylation of the cinnamoyl intermediate: ———, calculated for pK_A = 5.70; O, acetate, phosphate, and pyrophosphate buffers (μ = 0.1); ◇, tris buffer; D, carbonate buffer.

and conjugate basic components of the cinnamoyl intermediate, *viz.*

$$\text{cinnamoyl–XH} \overset{K_A}{\rightleftharpoons} \text{cinnamoyl–X}^{\ominus} + \text{H}^{\oplus}$$

the fraction present as the conjugate base at any pH is $f_B = (\epsilon_A - \epsilon_X)/(\epsilon_A - \epsilon_B)$, where ϵ_X is the observed extinction coefficient. The spectral results obtained are in accord with this simple protonic dissociation model. The pK_A of the conjugate acid under these conditions (25°, ionic strength 0.1 M) is 5.9 ± 0.1 pH.

If one considers the deacylation reaction (Fig. 1), it is apparent that the pH dependence of the rate is directly related to the protonic dissociation of the cinnamoyl intermediate. The deacylation rate equation is hence either

$$v = k[\text{cinnamoyl–X}^{\ominus}]$$

or (1)

$$k[\text{cinnamoyl–XH}][\text{OH}^{\ominus}]$$

A comparison of Fig. 3 with Table I suggests that the cinnamoyl intermediate in this reaction is N-formyl-O-cinnamoylhydroxylamine (III).

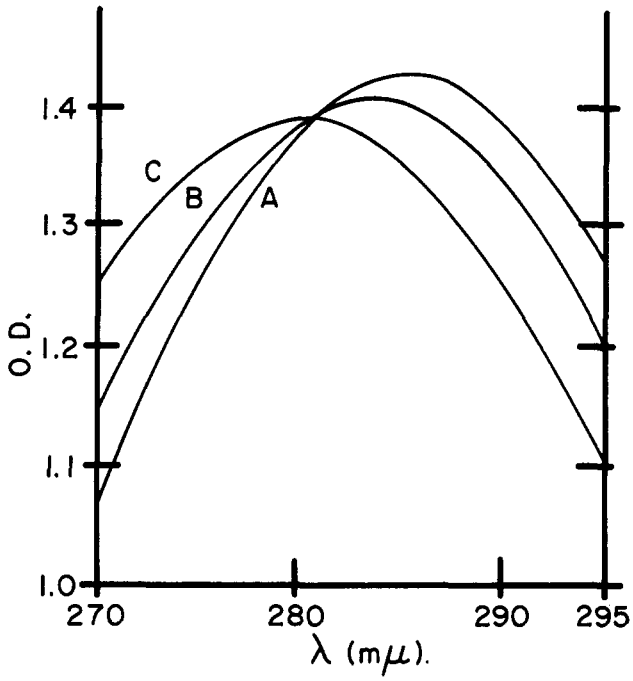
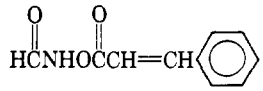


Fig. 3.—Ultraviolet spectra of the cinnamoyl intermediate (see text) in aqueous solutions; 1-cm. path; initial concentration of cinnamoylimidazole was 6.50×10^{-5} *M*, [FHA] = 2.33×10^{-3} *M*: curve A, pH 4.40 (acetate, μ = 0.10); curve B, pH 5.66 (acetate, μ = 0.10); curve C, pH 8.51 (pyrophosphate, μ = 0.10).



III

To verify this hypothesis, propionohydroxamic acid was prepared, and was allowed to react with either CI or cinnamoyl chloride. The same product was isolated in both cases. Treatment of the product with 6 *M* hydroxylamine–hydroxylammonium chloride at pH 6 resulted in the formation of 1 equiv. each of propionohydroxamate and cinnamohydroxamate as evidenced by the acidic $FeCl_3$ test and the ultraviolet spectrum of the cinnamoyl moiety. The product is presumably N-propionyl-O-cinnamoylhydroxylamine (see Experimental).

The ultraviolet spectra of this compound at various pH's are shown in Fig. 4. The spectra at low and high pH are virtually identical with the corresponding spectra shown in Fig. 2. Once more a plot of f_B *vs.* pH is in accord with a simple protonic dissociation model. In this instance, however, the diacylhydroxylamine is quite stable over the entire pH range of interest, and hence the pK_A' could be determined by direct titration, as well as spectrophotometrically. The spectroscopic and titrimetric results are mutually in accord. The pK_A' of the stable diacylhydroxylamine is pH 6.8 ± 0.1 at 25° and an ionic strength of 0.1 *M*.

On the Stability of Diacylhydroxylamines.—The structure of the unstable formyl intermediate having been established, it was of interest to examine the relative stability of other diacylhydroxylamines. Since the reaction of CI with N-acylhydroxamates could be followed spectrophotometrically by the change in the cinnamoyl spectrum, a variety of N-acyl-, O-cinnamoylhydroxamates was prepared by the addition of cinnamoylimidazole to an excess of acyl-N-hydroxylamine in phosphate buffer (pH 6.86, 0.05 *M*, 25°). In this way the O-cinnamoyl derivatives of N-acetyl-, N-propionyl-, N-hexanoyl-, and N-hippurylhydroxamic acid were studied. In every case, cinnamoylation of the acylhydroxamic acid was somewhat slower than cinnamoylation of FHA (Table II), and in no case was the extent of hydrolysis of the diacylhydroxylamine appreciable over 18–24 hr. at pH 6.86 and 25°. For example, the extent of hydrolysis, as estimated from the change in extinction at 290 mμ, was less than 3% over 20 hr. at pH 6.86 with N-acetyl-O-cinnamoylhydroxylamine. Increasing the pH to 9.2 (Tris or pyrophosphate buffer) increased the rate of deacylation of this latter compound less than tenfold.

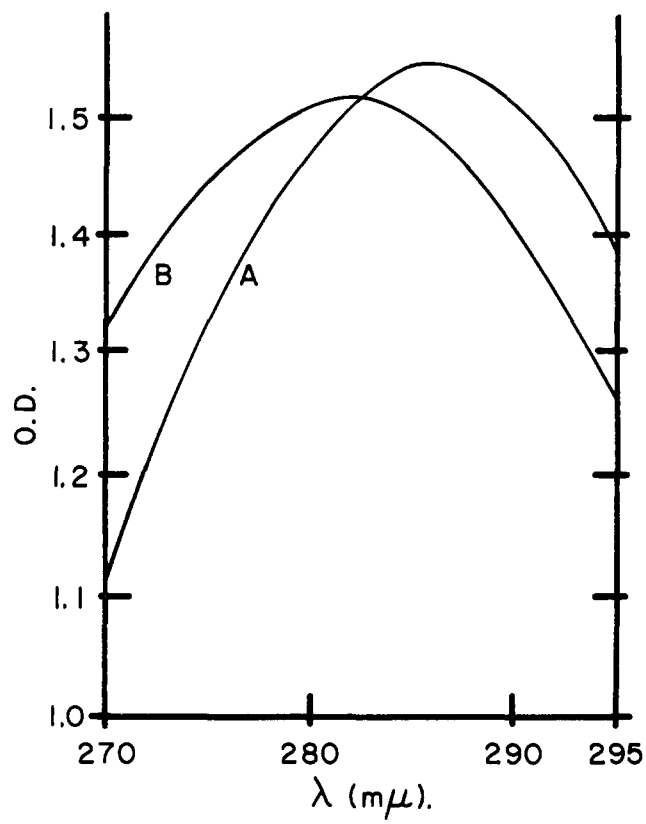


Fig. 4.—Ultraviolet spectra of N-propionyl-O-cinnamoylhydroxylamine (6.56×10^{-5} *M*) in aqueous solutions: curve A, pH 4.65 (acetate, μ = 0.10); curve B, pH 6.86 (phosphate, μ = 0.10).

TABLE II

RATES OF ACYLATION OF HYDROXAMATES BY CINNAMOYLIMIDAZOLE AT 25°, μ = 0.10

Acyl group	$k_{apparent} \times 10^{-2}$ (min.$^{-1}$ M^{-1}) pH 5.00	pH 6.86
Formyl	8.5	9.0
Acetyl	5.2	5.8
Hippuryl	3.83	4.10
Hexanoyl	2.48	2.80

The reaction of diacylhydroxylamines and other acyl derivatives with NH_2OH has been studied extensively by Jencks and by Jencks and Carriuolo.[4-6] On the basis of reactivity toward NH_2OH, the diacylhydroxylamines appear to be moderately reactive acyl derivatives (good acylating agents). Diacyl-

(4) W. P. Jencks, *J. Am. Chem. Soc.*, **80**, 4581 (1958).
(5) W. P. Jencks, *ibid.*, **80**, 4585 (1958).
(6) W. P. Jencks and J. Carriuolo, *ibid.*, **82**, 1778 (1960).

hydroxylamines are moderately stable toward hydrolysis. To examine further this distinction between hydrolysis and hydroxylaminolysis, the effect of NH_2OH and/or NH_3OH^+ on chromophoric diacylhydroxylamines was studied at pH 6. The results with N-formyl- and N-propionyl-O-cinnamoylhydroxylamine in 0.6 *M* hydroxylamine are illustrated in Fig. 5. Reaction takes place in at least two steps. First there is a rapid disappearance of the diacylhydroxylamine with the simultaneous appearance of a new absorbance maximum; λ_{max} of this intermediate is dependent on the nature of the N-acyl substituent (see Fig. 5). N-Propionyl and N-hexanoyl derivatives give rise to intermediates with λ_{max} 280 mμ, whereas N-formyl yields an intermediate with λ_{max} 276 mμ. The 280 mμ intermediate is probably cinnamoyl-O-hydroxylamine, since acyl-O-hydroxylamines have been identified as metastable intermediates in the reaction of NH_2OH with diacylhydroxylamines at this pH.[4] The nature of the intermediate with absorbance maximum at 276 mμ is under investigation and will be reported on in a later communication. The reaction of O-cinnamoylhydroxylamine with NH_2OH under the above conditions should yield cinnamohydroxamic acid (λ_{max} 274) exclusively.[4,5] This is indeed the final product of the reaction with N-propionyl-O-cinnamoylhydroxylamine (Fig. 5). The 276 mμ intermediate, however, yields cinnamate$^{\ominus}$ (λ_{max} 269.5) predominantly. The rate of cinnamate formation from the 276 mμ intermediate, moreover, is first order in NH_2OH at concentrations of NH_2OH in excess of 0.5 *M*. Thus the curious situation arises where NH_2OH is a catalyst (or activator) for an (apparent) hydrolytic reaction. Control reactions with cinnamoyl-N-hydroxamate, NH_2OH, and FHA under the above conditions showed no change in ultraviolet spectral properties over a 36-hr. period.

The Reaction of FHA with Other Acylating Agents.—The reaction of FHA with CI to yield the N,O-diacylhydroxylamine exhibits a pH–rate profile (Fig. 2) which is precisely the inverse of that found by Jencks in the reaction of aliphatic hydroxamates with *p*-nitrophenyl acetate.[5] In this latter reaction, the rate equation can be written as

$$v = k'[\text{ester}][\text{RCONHO}^-] \qquad (1)$$

whereas in the reaction of CI, the rate equation is apparently

$$v = k_0[\text{CI}][\text{HCONHOH}] \qquad (2)$$

In each instance, the conjugate acid or base does not appear to contribute significantly to the rate equation. Since CI is a moderately weak base, the possibility exists that the latter rate expression 2 is in actuality the kinetically equivalent expression 3.

$$v = k_1[\text{CIH}^+][\text{HCONHO}^-] \qquad (3)$$

The pK_A of CIH^+ is 3.65.[7] The pK_A' of FHA is 8.70 ± 0.05 under the conditions reported here (0.1 *M* ionic strength, 25°). According to formulation 3

$$v = \frac{k_1[\text{CI}]_{\text{total}}[\text{FHA}]_{\text{total}}[\text{H}^+]K_{\text{FHA}}}{\{K_{\text{CIH}} + [\text{H}^+]\}\{K_{\text{FHA}} + [\text{H}^+]\}} \qquad (4)$$

$$K_{\text{FHA}} = [\text{H}^+][\text{FA}^-]/[\text{FHA}]$$

$$K_{\text{CIH}} = [\text{H}^+][\text{CI}]/[\text{CIH}^+]$$

(7) M. L. Bender, G. R. Schonbaum, and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2562 (1960).

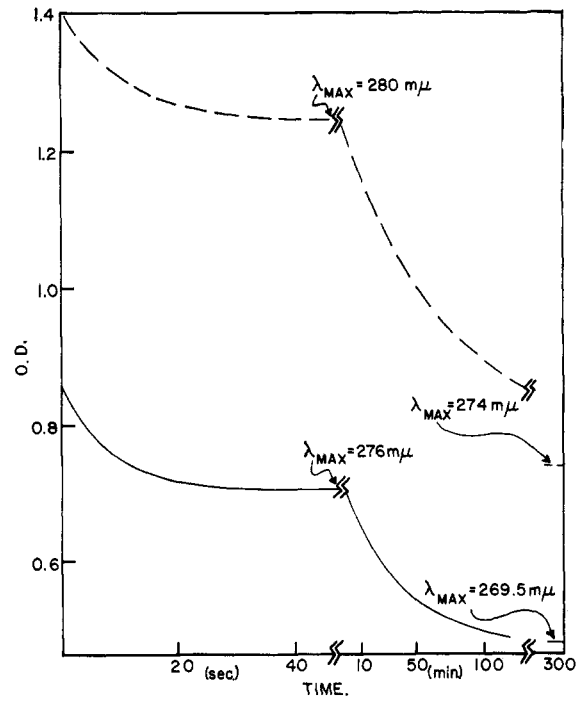


Fig. 5.—Reactions of N-formyl-O-cinnamoylhydroxylamine (———) and N-propionyl-O-cinnamoylhydroxylamine (– – – –) in 0.6 *M* hydroxylamine–hydroxylamine hydrochloride, pH 6.0, 25°, and μ = 0.3. Note that both the time and O.D. scales are common to both reactions. All kinetic measurements are at 290 mμ. Spectral scans were run at t = 3–4 min. and at t = 300 min. in both reactions.

whereas according to (1)

$$v = \frac{k_0[\text{CI}]_{\text{total}}[\text{FHA}]_{\text{total}}[\text{H}^+]K_{\text{CIH}}}{\{K_{\text{CIH}} + [\text{H}^+]\}\{K_{\text{FHA}} + [\text{H}^+]\}} \qquad (5)$$

Equations 4 and 5 are indistinguishable kinetically, both predicting the observed pH–rate profile. For this reason a nonionizable acylating agent, *p*-nitrophenyl acetate, was substituted for CI. In this instance, the acylation pH profile would be predicted to be the same as that reported by Jencks[5] if eq. 3 is correct, but should resemble that of Fig. 2 if eq. 2 obtains. The pH–rate profile for the formation of *p*-nitrophenylate (or *p*-nitrophenol) from *p*-nitrophenyl acetate and a large excess of FHA is shown in Fig. 2. The rate is clearly dependent on the concentration of hydroxamate anion and, hence, the rate equation for acylation of FHA by acylimidazoles is probably eq. 3. The specific rate of acylation, calculated according to the equation $v = k[\text{ester}][\text{HCONHO}^-]$, is $k = 3.4 \times 10^3$ min.$^{-1}$ M^{-1}, which is comparable to the specific rates of acylation of butyryl- and acetyl-N-hydroxamates by *p*-nitrophenyl acetate.[5]

Before drawing further conclusions from these experiments, it was necessary to establish that the aromatic acyl moiety (cinnamoyl) was of no special chemical significance. The reaction of acetylimidazole with FHA was therefore investigated. Acetylimidazole has an absorption maximum at 245 mμ. By analogy with acetyl esters of aliphatic alcohols, it was reasoned that N-formyl-O-acetylhydroxylamine would absorb maximally at a higher wave length than acetate + FHA, but at a lower wave length than acetylimidazole + FHA. This was indeed found to be the case. The initial acylation reaction in excess FHA could be con-

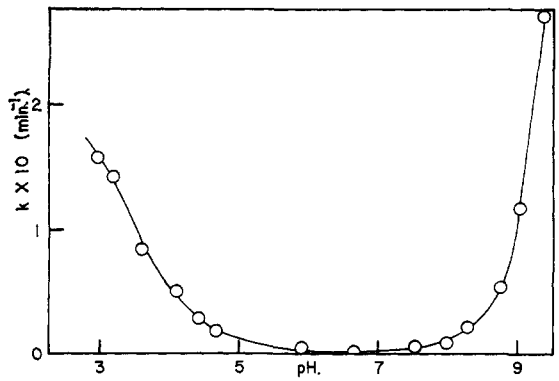


Fig. 6.—pH dependence of the hydrolysis of cinnamoylimidazole in 1.67% acetonitrile, 25°, μ = 0.1. The profile is very similar to that reported by Jencks and Carriuolo[8]: ———, calculated for pK_{CIH^+} = 3.66 and the kinetic parameters described in the text.

veniently followed at 260 mμ. The two rate constants at pH 6.86 (0.05 *M* phosphate) are $k_0 = k_{acylation} = 4.2 \times 10^3$ min.$^{-1}M^{-1}$, and $k_{deacylation} = 3.2 \times 10^{-2}$ min.$^{-1}$. Both constants are similar in magnitude to those obtained with cinnamoylimidazole and hence there is no reason to suspect any difference in mechanism between aliphatic and aromatic acylating agents.

The Reaction of FHA with Furoylimidazole and Furylacrylylimidazole.—In order to study both the effect of other acyl groups on the reaction rates and to investigate the spectral characteristics of the diacylhydroxylamine intermediates, two other acylimidazoles (furoyl- and furylacrylylimidazole) were prepared. Both acyl groups have strong ultraviolet absorption bands. Pertinent details of rate and spectra are recorded in Tables III and IV.

TABLE III

RATES OF ACYLATION AND DEACYLATION OF FHA BY VARIOUS ACYLIMIDAZOLES[a]

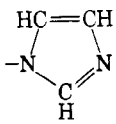
Acyl moiety	*k*(acylation), min.$^{-1}$ M^{-1} × 10^{-2}	*k*(deacylation), min.$^{-1}$ × 10^2	pK_A of corresponding carboxylic acid
Cinnamoyl	9.0	1.85	4.45
Furylacrylyl	8.3	2.15	4.5
Acetyl	42	3.2	4.75
Furoyl	19	24	3.12

[a] pH 6.86 (phosphate buffer, 0.05 *M*), 25°.

TABLE IV

SPECTRAL PROPERTIES OF CHROMOPHORIC ACYL DERIVATIVES[a]

(R—C(=O)—X)

	X = 	-OCH$_3$	-ONHCHO	-O$^\ominus$
Cinnamoyl	2.55 (307)	2.21 (279)	2.33 (285)	2.03 (269.5)
Furylacrylyl	3.00 (340)	2.65 (308)	2.75 (315)	2.5 (293)
Acetyl	0.16 (245)	...	...	...
Furoyl	1.5 (284)	1.1 (255)	1.2 (255)	1.1 (245)

[a] ϵ_{max} × 10^{-4} (O.D., M^{-1} cm.$^{-1}$) (λ_{max} in mμ in parentheses).

Furoic acid is a considerably stronger acid than either acetic or cinnamic acid. The rates of nucleophilic reactions of acyl derivates (*e.g.*, saponification or aminolysis) are usually highly sensitive to the electron-withdrawing power of the acyl substituent, and hence correlate with the pK_A's of the corresponding carboxylic acids. In the acylation of hydroxamate anion, the true rate constant at optimum pH is

$$k_1 = k_{obsd} \times (K_{AcIH^\oplus}/K_{FHA})$$

Hence k_1, and not k_{obsd}, should be correlated with the pK_A' of the corresponding carboxylic acid. On this basis, acylation with furoylimidazole is probably a much faster process than acylation with any of the other acyl derivatives listed in Table III, since the pK_A' of this acylimidazolium cation is probably lower than that of the others. The pK_A of acetylimidazolium$^\oplus$ (3.6)[8] is about the same as that of cinnamoylimidazolium$^+$ and hence acetylation is about fivefold faster than cinnamoylation. In deacylation, the observed maximum rate constant is straightforwardly interpretable from eq. 10 (below) and, as is indicated in Table III, the rates correlate well with the pK_A's of the corresponding carboxylic acids.

The spectra of furylacryl derivatives merit special attention owing to both their high extinctions and their wave lengths of the maximum absorption. Since many potential model catalysts absorb ultraviolet radiation in the region of *ca.* 280 mμ and below, a set of acyl derivatives with high extinctions in the region above 300 mμ are of great utility in the study of acylation and deacylation reactions, provided the spectra are sensitive to changes in the acyl–X bond. The furylacrylyl derivatives conform to these requirements better than any acyl derivatives thus far tested.[9] These requirements are of special importance in the study of acyl–enzyme reactions[10] since proteins absorb ultraviolet radiation strongly in the region 260–300 mμ owing to the presence of tyrosine and tryptophan residues.

FHA Catalysis.—In the reaction of FHA with acylating agents in aqueous solution, the intermediate N-formyl-O-acylhydroxylamine is unstable, ultimately yielding the corresponding acylate. The reactions of other N-acylhydroxamates with acylating agents (reported herein) yield stable diacylhydroxylamines.

The unusual nucleophilicity of hydroxamate anion in aqueous solution has been reported previously for reactions involving nitrophenyl esters,[5] acyl halides and anhydrides[11] and organofluorophosphates.[11–14] The specific rate for the reaction

acylimidazolium$^\oplus$ + hydroxamate$^\ominus$ ⟶ O-acylhydroxamate + imidazole

is very much greater than would be anticipated even on the basis of the previously reported unusual nucleophilicity of hydroxamate anion.[6] A comparison of the rates of nucleophilic attack of hydroxamate and hydroxide anions on CI is illustrative. The pH dependence of the rate of CI hydrolysis in aqueous solution is shown in Fig. 6. From this plot it is evident that at least three rate expressions are pertinent, *viz.*

(8) W. P. Jencks and J. Carriuolo, *J. Biol. Chem.*, **234**, 1272 (1959).

(9) Various nitrocinnamoyl derivatives were also tested. Although these derivatives exhibit intense absorption spectra above 320 mμ, their spectra are relatively insensitive to the nature of the acyl–X bond in this wave length region.

(10) S. A. Bernhard, S. J. Lau, and H. Noller, *Biochemistry*, submitted for publication.

(11) H. L. Yale, *Chem. Rev.*, **33**, 209 (1943).

(12) B. E. Hackley, Jr., R. Plapinger, M. Stolberg, and T. Wagner-Jauregg, *J. Am. Chem. Soc.*, **77**, 3651 (1955).

(13) R. Swidler, R. E. Plapinger, and G. M. Steinberg, *ibid.*, **81**, 3271 (1959).

(14) M. A. Stolberg and W. A. Mosher, *ibid.*, **79**, 2618 (1957).

$$v = k[\mathrm{CI}][\mathrm{OH}^-] \quad (6)$$

$$v_{\mathrm{i}} = k'[\mathrm{CI}][\mathrm{H_2O}] \quad (7a)$$

and/or

$$v_{\mathrm{ii}} = k''[\mathrm{CIH}^+][\mathrm{OH}^-] \quad (7b)$$

$$v_{\mathrm{iii}} = k'''[\mathrm{CIH}^+][\mathrm{H_2O}] \quad (8)$$

and/or

$$v_{\mathrm{iv}} = k^{\mathrm{iv}}[\mathrm{CI}][\mathrm{H_3O}^+]$$

A plot of the apparent first-order rate constant *vs.* $[\mathrm{OH}^-]$ is linear in the range pH 7–9.5 and indicates that expression 6 predominates above pH 8.5 and that $k = 5.6 \times 10^3\ M^{-1}$ min.$^{-1}$. The intercept of this plot yields the observed minimum rate constant, $k_{\mathrm{min}} = 2.5 \times 10^{-3}$ min.$^{-1}$. This minimum rate is predominately the sum of the two pH independent and kinetically indistinguishable expressions 7a and 7b, *viz.*

$$v_{\mathrm{min}} = k_{\mathrm{app}}[\mathrm{CI}] = k'[\mathrm{CI}][\mathrm{H_2O}] + k''[\mathrm{CIH}^+][\mathrm{OH}^-]$$
$$\cong [\mathrm{CI}]_{\mathrm{total}}k'[\mathrm{H_2O}] + k''K_{\mathrm{w}}/K_{\mathrm{CIH}}$$

since $[\mathrm{H}^+] << K_{\mathrm{CIH}}$ in this pH range. If expression 7b predominates over 7a, $k'' \gtrsim 5.5 \times 10^7$ min.$^{-1}$ M^{-1} or, in other words, $k'' \sim 10^4 k$. This maximal value of k'' is within an order of magnitude of that reported by Wolfenden and Jencks[15] for hydroxyl ion attack on acetyl-N-methylimidazolium$^{\oplus}$. An estimate can be made of the maximum value (k'_{acyl}) for the acylation of FHA^- by neutral CI from the results illustrated in Fig. 2. At pH 9.10, 0.1 M ionic strength, and 25°, the fraction of FHA present as the anion is 0.68. The rate of acylation should therefore be 0.32 v_{opt} if eq. 3 is the complete rate expression at pH 9.10. This is, in fact, the observed rate at pH 9.10. If allowance is made for the error in the determination of v and the pK_{A} of FHA, a contribution of 10% to the total rate expression at pH 9.10 from the term

$$v'_{\mathrm{acyl}} = k'_{\mathrm{acyl}}[\mathrm{CI}][\mathrm{FHA}^-]$$

is improbable but not impossible. Admitting of this possibility, $k'_{\mathrm{acyl}} \lesssim 6\ M^{-1}$ min.$^{-1}$. For the reaction

$$\mathrm{CIH}^+ + \mathrm{FHA}^- \longrightarrow \text{N-formyl-O-cinnamoylhydroxylamine} + \text{imidazole}$$

$k_{\mathrm{acyl}} = 1 \times 10^8\ M^{-1}$ min.$^{-1}$. The ratio $k_{\mathrm{CIH}^+}/k_{\mathrm{CI}}$ is more than 1.6×10^3 greater for the nucleophilic attack by hydroxamate anion than for the nucleophilic attack by hydroxide anion. The high bimolecular rate constant for the nucleophilic attack of hydroxamate anion on acylimidazolium cation cannot be ascribed solely to charge–charge interactions, nor can the very large bimolecular rate constants for the nucleophilic attack by hydroxamate anion on uncharged acylating agents be ascribed solely to the anionic nature of the nucleophile. The rate of nucleophilic attack by hydroxamate is to only a small extent dependent on the nature of the particular hydroxamate anion. With cinnamoylimidazole as substrate there is a difference of less than a factor of four between the highest specific rate (FHA) and the lowest (*n*-hexanohydroxamate) reported herein. These relatively small differences in rate are, perhaps, not surprising in light of the small differences in the pK_{A}'s of the corresponding hydroxamic acids. The pK_{A}'s of FHA, hippurohydroxamic acid, and hexanohydroxamic acid are 8.7, 8.95, and 9.1, respectively. The pK_{A}'s of the corresponding carboxylic acids are 3.7, 3.5, and 4.8, respectively.

(15) R. Wolfenden and W. P. Jencks, *J. Am. Chem. Soc.*, **83**, 4390 (1961).

D_2O Experiments.—The optimum rates of the acylation and deacylation reactions of FHA with CI were studied in D_2O solvent. The rate of acylation of FHA shows a small deuterium isotope effect, the rate being 1.4-fold faster in H_2O than in D_2O. This may arise from two factors: (1) The optimum rate of acylation is $v_{\mathrm{opt}} = k(K_{\mathrm{FHA}}/K_{\mathrm{CIH}})$. If the ratio of the two acid dissociation constants is greater in H_2O than in D_2O, the optimum rate will be faster in H_2O. (2) The acylation reaction probably occurs in two steps: addition of hydroxamate$^{\ominus}$ to the carbonyl carbon, followed by elimination of imidazole from the tetrahedral adduct. Proton transfer may occur in the latter process and may be partially rate controlling. The rather small decrease of the rate of acylation in D_2O suggests that this process is only partially rate controlling. The rate of deacylation of the diacylhydroxylamine, on the other hand, shows no D_2O isotope effect whatsoever; the maximum rate of deacylation is the same in both H_2O and D_2O. This result is somewhat surprising in light of the notable deuterium isotope effects in the solvolysis of carboxylic acid derivatives, particularly in reactions involving either neutral H_2O or $OH^{\ominus}$. We therefore further investigated the nature of the deacylation reaction as described below.

The Mechanism of Deacylation.—In contrast to the small differences in acylation rates, the rate of release of acylate ion from an N,O-diacylhydroxylamine varies markedly depending on whether or not the N-acyl substituent is a formyl residue. At pH 6.86 and 25°, the rates of deacylation of N-propionyl-, or N-hexanoyl-, or N-hippuryl-O-cinnamoylhydroxylamine are all too slow to be determined by the usual kinetic methods employed herein. Incubation of such aqueous solutions for 2 days at 25° resulted in the appearance of measurable amounts of cinnamate (*ca.* 10–40%, estimated spectrophotometrically). However, the amount of N-hydroxamate, as determined either from the $FeCl_3$ test (see Experimental) or from the rate of deacylation of cinnamoylimidazole, is never stoichiometric with the amount of cinnamate formed. This suggests that some cinnamate is formed *via* another pathway, presumably by a Lossen rearrangement (eq. 9).[16]

$$\mathrm{R{-}C(=O){-}\bar{N}{-}O{-}C(=O){-}CH{=}CH{-}C_6H_5} \longrightarrow \mathrm{RNCO} + \text{cinnamate} \quad (9)$$

Allowing for possible errors in rate measurements, the rate of deacylation of N-formyl-O-cinnamoylhydroxylamine exceeds that of other N-acyl-O-cinnamoylhydroxylamines by at least a factor of 10^3. That this unique situation does not arise from the greater electron-withdrawing power of the formyl moiety is indicated by the similar pK_{A}'s of N-formyl- and N-hippuryl-O-cinnamoylhydroxylamines (5.9 and 6.0, respectively). The pK_{A} of the corresponding N-propionyl derivative is considerably higher (6.8).

The deacylation results presented above do not permit discrimination between hydroxide attack on the uncharged diacylhydroxylamine, and direct deacylation or attack of water on the corresponding anion (eq. 1).

(16) (a) A. Lossen, *Ann.*, **252**, 170 (1889). For more recent discussions, see (a) ref. 20; (b) F. Mathis, *Bull. soc. chim. France*, 1953, D9; (c) J. Hine, "Physical Organic Chemistry," 2nd ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p. 335.

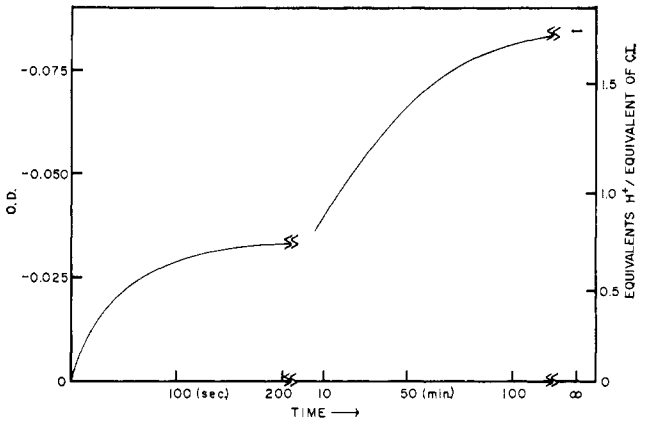


Fig. 7.—The reaction of 2.0×10^{-4} mequiv. of CI in 3 ml. of FHA (2.0×10^{-3} M) originally at pH 8.00, in the presence of $\sim 2 \times 10^{-5}$ M brom thymol blue indicator. Kinetics were followed at 620 mμ. The right-hand ordinate was calibrated by the addition of standard HCl ($0.5–2.0 \times 10^{-4}$ mequiv.) to the same solution.

N-Methylformohydroxamic acid was therefore prepared, and the reaction with cinnamoylimidazole was studied as a function of pH as described previously with FHA. Results are given in Table V.

TABLE V

RATES OF ACYLATION AND DEACYLATION OF N-METHYL FORMOHYDROXAMATE BY CINNAMOYLIMIDAZOLE

pH	$k_{acylation} \times 10^{-2}$, min.$^{-1}$ M^{-1}	$k_{deacylation} \times 10^{2}$, min.$^{-1}$
6.86[a]	4.6	0.13
8.91[b]	1.06	1.4

[a] Phosphate buffer, $\mu = 0.1$, 25°. [b] Pyrophosphate buffer, $\mu = 0.1$, 25°.

The rate of hydrolysis of N-formyl-O-cinnamoyl-N-methylhydroxylamine is not particularly rapid even at pH 9. Moreover, the results (Table V) imply that the hydrolytic rate of the N-methyl derivative at pH 6.86 is caused largely by attack either by neutral water or by oxonium ion rather than by hydroxyl ion. This strongly suggests that the deacylation of N-formyl-O-acylhydroxylamine proceeds according to eq. 10. However, it might be argued that the presence

$$v = k[\text{diacylhydroxylaminate}^-] \qquad (10)$$

of an N-methyl group in the diacylhydroxylamine sterically prevents the approach of an otherwise rapidly attacking hydroxyl ion to the O-acyl carbonyl carbon, and hence analogies based on the behavior of the N-methyl derivative may not necessarily be valid.

The nature of products other than cinnamate, following the deacylation of N-formyl-O-cinnamoylhydroxylamine, were investigated preliminarily in two ways: (1) 1.1 equiv. of CI was added to 1.0 equiv. of FHA (pH 5.7, acetate buffer) and the reaction mixture was allowed to stand 24 hr. at room temperature. After incubation, the ultraviolet-absorbing product was exclusively cinnamate ion. The resulting solution gave a negative hydroxamic acid test (less than 0.01 equiv. of FHA). A control sample of FHA remained unchanged over this time period.

(2) 1.0 equiv. of CI was treated with 1.0 equiv. of FHA and the reaction was followed spectrophotometrically as described previously. The concentrations of FHA and CI (10^{-3} M) were such that acylation was essentially complete long before a significant fraction of CI could have hydrolyzed. When the deacylation reaction was virtually complete, a second equivalent of CI was added. No significant increase in CI disappearance over the blank rate could be noted following this second addition.

Both these experiments demonstrate unambiguously that FHA is destroyed in the stoichiometric conversion of acylimidazole to acylate ion. A plausible mechanism for this destruction is the elimination of acylate from the diacylhydroxylamine anion (eq. 11), a process in some way analogous to the Lossen rearrangement.

$$\text{H—C(=O)—}\overset{-}{\text{N}}\text{—O—C(=O)—R} \longrightarrow \text{H}^+ + \text{CNO}^- + \text{RCO}_2^- \qquad (11)$$

To test this hypothesis, the number of equivalents of protons released upon deacylation of the diacylhydroxylamine anion was measured near pH 8 where all dissociable species (cinnamic acid, diacylhydroxylamine, and cyanic acid) are essentially completely dissociated. Starting with CI (1 equiv.) and FHA (30 equiv., pH 8.00) the proton release upon acylation and deacylation was followed by measurement of the change of absorbance of an acid–base indicator (brom thymol blue) as is described in the Experimental. The excess FHA functioned as a weak buffer. The results are shown in Fig. 7. Over the range of pH change of this experiment, the rate of change of the basic component of the indicator with $[H^+]$, $(d[I]/d[H^+])$ is nearly linear. Hence the nearly first-order rates of proton release could be compared with the directly measured acylation and deacylation rates. Both rates agree, to within the limits of estimate of the indicator method (±5%), with the previously determined acylation and deacylation rates; 0.75 equiv. of H^+ was released on acylation (calcd. 0.77 based on pK_A's of 7.0 and 8.7 for imidazolium$^+$ and FHA, respectively) and 1.0 equiv. of H^+ was released on deacylation. These results are quantitatively consistent with the *direct* release of cyanate ion upon deacylation. That cyanate ion is the direct product of deacylation was further established by the stoichiometric reaction of 1 equiv. of CI with 1 mole of FHA at pH 8.5 (0.1 M pyrophosphate buffer). After several hours the reaction mixture was acidified to pH 3 and warmed. At this pH, cyanic acid is quantitatively converted to NH_4^+ and CO_2. One equivalent of NH_4^+ (by Nessler test) was found per initial equivalent of FHA. A control reaction containing FHA and treated identically except for the omission of cinnamoylimidazole was NH_4^+ negative.

Enzyme Models.—The formohydroxamic acid promoted formation of acylate$^-$ from acylating agents is not catalytic, since the hydroxamic acid is stoichiometrically destroyed in the process. Nevertheless, certain aspects of these studies bear on the mechanism of catalysis by proteolytic enzymes. Primarily, consideration should be given to the high rate of acylation of hydroxamate by acylimidazolium. This acylation rate is within a few orders of magnitude of the rate of a diffusion-controlled process.[17] Moreover, the second-

(17) M. Eigen and L. de Mayer in "Techniques in Organic Chemistry," Vol. VIII, Part 2, A. Weissberger, Ed., Academic Press, Inc., New York, N. Y., 1963.

order specific rate for this process exceeds, somewhat, the second-order specific rate of reaction of acylimidazolium with OH^-, a result in striking exception to the usual (Brønsted) relationship between base strength and nucleophilicity but analogous to that found in the proteolytic enzymes, where a group with pK_A' near neutrality is apparently involved in the rapid formation of acyl–enzyme. The chemically specific reaction of a protonated acylating reagent with an unusual nucleophile is suggestive of an enzymic mechanism whereby a protonated acyl derivative reacts with an unusual nucleophile at the enzyme site.[2]

Experimental

The Reaction of N-Carbobenzyloxy-β-methyl-L-aspartyl-L-serylamide with Hydroxylamine.—N-Carbobenzyloxy-β-methyl-L-aspartyl-L-serylamide (METCASA) was employed in the reaction with hydroxylamine. In the presence of low concentrations of OH^- ($\sim 10^{-6}$ M) it is very rapidly converted to the corresponding imide (see eq. 1). In order to avoid the possibility of formation and cleavage of the imide to a mixture of α- and β-hydroxamates (see eq. 2), hydroxylaminolysis was run a ta pH somewhat below the pK_A of hydroxylammonium cation (pK_A 6.1),[18] usually at an apparent pH of 5.6 (in 50 volume % aqueous dioxane–1.0 M hydroxylamine–0.1 M METCASA). At this pH the formation of hydroxamate, as measured by the optical density at 520 mμ of a $FeCl_3$ complex, occurred more rapidly than did the formation of imide II, in the same solvent and at the same ionic strength, in the absence of hydroxylamine. The hydroxylaminolysis reaction can be shown to be complex. A peptide product can be isolated from the initial system after 4 hr. which is not the imide, N-hydroxamate, METCASA, nor any hydrolytic product of METCASA. Elemental analysis, low solubility in aqueous and high solubility in nonpolar solvents, and the spectral characteristics of the product of reaction of this compound with cinnamoylimidazole suggest that this is the corresponding aspartyl-β-O-hydroxylamine derivative of METCASA. With increasing reaction time, the products become increasingly water-soluble and give increasingly positive hydroxamic acid tests. After 3 days incubation at room temperature, water-soluble products with quantitatively reproducible catalytic properties toward acylating agents can be isolated. At intermediate times (1–2 days) mixtures of products with varying catalytic properties can be isolated.

Isolation of Formohydroxamic Acid from the Hydroxylaminolysis Reaction.—N-Carbobenzyloxy-β-methyl-L-aspartyl-L-serylamide (0.8 g., 0.002 mole) was dissolved in 10 ml. of dioxane and 10 ml. of 2 M aqueous hydroxylamine hydrochloride adjusted to pH 5.6 with NaOH was added. The reaction mixture was allowed to stand in the dark for 3 days at 20°. Periodic removal of aliquots indicated that hydroxamate formation was complete at this time; 80 cc. of water was then added, whereupon a slight precipitate (hydroxamate negative) formed. The mixture was filtered and the filtrate extracted in three steps with a total of 2000 ml. of ethyl acetate; 90% of the hydroxamate-positive material remained in the aqueous phase. The aqueous phase was evaporated to dryness *in vacuo* at 40° with a flash evaporator. The remaining isolation steps were hindered by the apparent strong absorption of FHA to the salts (NaCl and $NH_3OH^+Cl^-$). Isolation of FHA was achieved by successive extractions with solvents in which the salts were progressively less soluble, *viz.*, dimethylformamide, dioxane, and hot (50°) acetonitrile. After each extraction the solvent was removed by flash evaporation. Following evaporation of the acetonitrile extract to dryness, the residue was extracted with 100 ml. of anhydrous ethyl acetate (under reflux) and the hot extract was filtered. On cooling to room temperature, large crystals (plates) formed. An additional yield of crystals was obtained by cooling to −20°. The crystals were washed with cold anhydrous ethyl acetate and dried *in vacuo*; yield 60–85 mg. (0.0010–0.0014 moles) for three different preparations; m.p. 77.5°; m. m.p. with a sample of FHA prepared by hydroxylaminolysis of ethyl formate, 77.5°; yield of hydroxamate recovered from the crude reaction mixture (by $FeCl_3$ test) 50–70%. *Anal.* Calcd. for CH_3O_2N: C, 19.7; H, 4.9; N, 23.0; O, 52.5. Found: C, 20.0; H, 4.9; N, 23.1; O, 52.0; Cl^+, <0.1.

When the same initial system of reactants as above is maintained at pH 9.0 in a "pH-stat," a simpler reaction appears to ensue. N-Hydroxamate increases rapidly with time, reaching a stable level of about 1 mole/mole METCASA within 1 hr. An apparently homogeneous product (a weak acid, pK_A 8.8, neut. equiv. 390) containing one carbobenzyloxy residue (by ultraviolet spectral analysis) and 1 equiv. of N-hydroxamate (by $FeCl_3$ test) per equivalent of acid can be isolated by evaporation of the dioxane under reduced pressure and extraction of the residual aqueous solution with ethyl acetate. A sharp melting point is suggestive of a single isomer, most probably N-carbobenzyloxyaspartyl(β-hydroxamate)serinyamide. This compound was found to react rapidly with acylating agents; with cinnamoylimidazole, a stable product was rapidly formed and was identified spectrophotometrically as the N-peptidyl-cinnamoylhydroxylamine.

Preparation of FHA by Direct Hydroxylaminolysis.—Sodium methoxide (0.5 mole) was dissolved in 400 ml. of absolute ethanol. The solution was maintained at room temperature in a water bath; 0.5 equiv. (84 g.) of $(NH_3OH)_2SO_4$ dissolved in 100 ml. of H_2O was slowly added with continuous, vigorous stirring. After 15 min. of stirring, the solution was cooled to 2° and the Na_2SO_4 was removed by filtration; 30 ml. of ethyl formate (Matheson Coleman and Bell) was added to the filtrate, the solution was allowed to stand for 4 hr. at room temperature and then carefully titrated to an *apparent* pH of 4.0 with 9 N H_2SO_4. The mixture was again cooled to 2° and excess $(NH_3OH)_2SO_4$ was removed by filtration. The filtrate was evaporated to a viscous oil at room temperature. The solid mass was extracted with three 50-ml. portions of hot (~50°) acetonitrile (reagent grade). Upon cooling to room temperature, large crystals (plates) of formohydroxamic acid began to appear. Further cooling to 2° resulted in an additional yield of FHA. The crystals were recrystallized from hot anhydrous reagent grade acetonitrile; m.p. 77.5° (cor.), yield 8 g. (40%); equiv. wt. by potentiometric titration, 61.0; sulfate content <0.1% *Anal.* Calcd. for CH_3NO_2: C, 19.6; H, 4.9; N, 23.0; O, 52.4. Found: C 20.1; H, 5.0; N, 23.0; O, 51.8.

Other Materials —Hippuryl, hexanoyl, cinnamoyl, acetyl, and propionyl hydroxamates were prepared by alkaline hydroxylaminolysis of the corresponding methyl or ethyl esters in ethanolic hydroxylamine. Melting points agreed with previously cited values. Titration equivalents agreed with the calculated molecular weights to within ±1%.

Cinnamoylimidazole was prepared by the method of Schonbaum, Zerner, and Bender.[19]

2-Furylacrylyl chloride was prepared by reacting 0.02 mole of furylacrylic acid (Aldrich Chemical Co.), twice recrystallized from anhydrous redistilled benzene with 0.025 mole of redistilled thionyl chloride at room temperature for 4 hr. under anhydrous conditions.

Volatile material was removed from the resultant dark liquid by flash evaporation *in vacuo* employing a solid NaOH trap. The residue was extracted with cyclohexane, partially decolorized with Norit, filtered, and the cyclohexane removed by flash evaporation. The resultant acyl halide (m.p. ~20°) was employed without further purification for the preparation of furylacrylylimidazole.

2-Furylacrylylimidazole was prepared from the acyl halide following (precisely) the method of preparation of CI.[19] The crystalline product, m.p. 110°, could be decolorized significantly with Norit in hot cyclohexane during recrystallization.

Acetyl- and furoylimidazole were prepared from the acyl halides (Matheson Coleman and Bell) as described previously.[20,21]

Formo(N-methyl)hydroxamic acid was prepared similarly to FHA but starting with N-methylhydroxylamine hydrochloride (Allied Chemical Co.) suspended in absolute ethanol and neutralizing with sodium methoxide in ethanol to remove sodium chloride. The oily product is soluble in most solvents (*e.g.*, H_2O, chloroform, dioxane, ether, and, to an appreciable extent, *n*-hexane). A solid could be isolated (from a $CHCl_3$–*n*-hexane mixture) at −20°, which formed an oil on warming to room temperature. When an aqueous solution 10^{-4} M in the hydroxamic acid (by titration) was treated with 0.8 equiv. of cinnamoylimidazole at pH 6.86 (phosphate), the ultraviolet spectrum of the

(18) S. A. Bernhard, W. C. Coles, and J. F. Nowell, *J. Am. Chem. Soc.*, **82**, 3043 (1960).

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

(20) J. H. Boyer, *J. Am. Chem. Soc.*, **74**, 6274 (1952).

(21) M. Caplow and W. P. Jencks, *Biochemistry*, **1**, 833 (1962).

reaction product was virtually identical with that of the protonated (uncharged) form of N-propionyl-O-cinnamoylhydroxylamine. This spectrum was independent of pH in the pH range 4–9.

Preparation of N-Propionyl-O-cinnamoylhydroxylamine.—Two methods of preparation of this compound were employed—the reaction of N-propionylhydroxamic acid with cinnamoyl chloride in anhydrous pyridine, and the reaction of the same hydroxamic acid with cinnamoylimidazole in anhydrous dioxane. Following reaction (which appeared to be nearly instantaneous in both instances) the organic solvent was removed by a flash evaporation *in vacuo*. The residual solid was extracted with acetate buffer, pH 5.5, and with H_2O. The crude sticky solid was extracted with ethyl acetate, and the ethyl acetate extract was evaporated to dryness. The resultant solid was recrystallized from hot water–dioxane; m.p. 119° (cor.); neut. equiv. calcd. 219, found 221.

Methods. pH Measurements.—All potentiometric titrations reported herein were carried out in 0.1 *M* KCl as solvent, employing KOH as titrant. A Radiometer precision pH meter (Model 25SE) was used throughout. All buffers were calibrated against National Bureau of Standards pH standards employing a Radiometer Model pH m-4 precision pH meter. All pH measurements were carried out in thermostated water-jacketed vessels maintained at 25.0 ± 0.1°.

Spectral Measurements.—A Cary Model 14 recording spectrophotometer was used in all measurements reported herein. Inner and outer cell compartments were thermostated at 25.0 ± 0.1°.

Kinetics of acylation and deacylation of cinnamoylimidazole were followed essentially according to the methods of Bender, Schonbaum, and Zerner.[3] The hydroxamates were all transparent above 270 mμ at pH 6.86 or below at the concentration levels employed, obviating the need for highly precise matching of reference and sample. At high pH (near or above the pK_A') significant absorption was noted below 280 mμ (although no new absorption peaks were detected above 230 mμ) and precise blanks were prepared in such experiments as well as in all experiments involving furoyl- or acetyl-acylating agents.

D_2O Experiments.—Reactions in D_2O were compared to reactions in H_2O by dilution of concentrated aqueous phosphate buffer with either D_2O or H_2O. The final concentration of D_2O was 97% in every D_2O experiment. The phosphate buffers maintained the pH (or pD) such that all measured rates were within 5% of the optimal rates (in the respective solvents) at this temperature.

$FeCl_3$ Test for Hydroxamates.—A stock solution containing 1.33% $FeCl_3$, 0.013 *M* HCl, and 0.53 *M* monochloracetic acid was used in all hydroxamate tests. This solution has the advantages of yielding stable (nonfading) ferric–hydroxamic acid complexes which are insensitive to dilution over the dilution range employed. In typical experiments, $10–500 \times 10^{-3}$ ml. of sample was mixed with 3 ml. of stock solution and the optical density read at 520 mμ with a Beckman Model DU spectrophotometer. Aliphatic acylhydroxamates all gave the same extinction, *viz.*, 1.09×10^3 O.D./cm. *M*. Formohydroxamate gave a considerably lower extinction (0.85×10^3).

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[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACEUTICAL CHEMISTRY OF THE UNIVERSITY OF WISCONSIN, MADISON 6, WIS.]

Buxus Alkaloids. III.[1] The Structure of Cyclobuxine

BY KEITH S. BROWN, JR.,[2] AND S. MORRIS KUPCHAN[3]

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Physical and chemical evidence have led to the assignment of structure Ia (3,20-bis(methylamino)-4-methylene-14-methyl-9,19-cyclopregnan-16-ol) to cyclobuxine, the major alkaloid of the acetone-insoluble portion of the bases of *Buxus sempervirens* L. The gross skeletal structure for cyclobuxine was indicated by the structures suggested for the products of selenium dehydrogenation (naphthalenes III and VI, phenanthrene V, and anthracene IV); dehydrogenation of decyclized cyclobuxine (XIa) gave only phenanthrene-related material. Key degradation products in the structure assignment were a conjugated diene (VIIIa) produced by Hofmann degradation; a mixture of *cis*- and *trans-cisoid* cyclopentenones (XVIa and b) arising from oxidation followed by facile elimination of methylamine; and the product from the ozonolysis of cyclobuxine followed by alkali treatment of the thus resultant α-aminoketone, the diosphenol XIIIa, which showed additional conjugation in the ultraviolet spectrum.

Extracts of *Buxus sempervirens* L. have been used since ancient times in the treatment of a wide variety of diseases, including malaria and venereal disease.[4] More recently an alkaloidal extract of the plant has been reported to possess antitubercular properties.[5] Previous chemical studies have indicated the multicomponent nature of the alkaloidal extract.[4,6–8] We have isolated four alkaloids from the acetone-insoluble portion of the strong bases of *B. sempervirens*, and have elucidated the structures of the three major bases. This paper reports the structure of the major alkaloid, cyclobuxine (Ia), the first steroidal alkaloid recognized to contain a cyclopropane ring and the first having a substitution pattern at C-4 and C-14 which is intermediate in the biogenetic scheme between lanosterol- and cholesterol-type steroids.[9] The configuration of cyclobuxine[10] and the constitutions of the remaining two alkaloids[11,12] will be presented in future communications.

Cyclobuxine (Ia), $C_{25}H_{42}ON_2$, demonstrated infrared bands for a terminal methylene (6.09 and 11.20 μ) and n.m.r.[13] peaks for the terminal methylene in an elec-

(1) Parts I and II: Keith S. Brown, Jr., and S. Morris Kupchan, *J. Am. Chem. Soc.*, **84**, 4590, 4592 (1962). The material presented in this paper was first outlined in Part I.

(2) National Science Foundation Cooperative Predoctoral Fellow in Chemistry, 1960–1962.

(3) To whom inquiries concerning this paper should be directed. This investigation was supported in part by research grants from the National Institutes of Health (H-2952 and CY-4500).

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