(32) I. H. Sadler, *J. Chem. Soc. B*, 1024 (1969).
(33) E. T. Scafe, J. Herman, and G. R. Bond, Jr., *Anal. Chem.*, **19**, 971 (1947).
(34) Methylenetriphenylphosphorane was prepared salt-free in three different ways: (a) the sodium amide/liquid ammonia method;[3] (b) the sodium amide in refluxing THF method [R. Köster, D. Simić, and M. A. Grassberger, *Justus Liebigs Ann. Chem.*, **739**, 211 (1970)]; and (c) the sodium hydride in THF method, which seemed in our hands only successful in the case of methyltriphenylphosphonium bromide [H. Schmidbaur, H. Stühler, and W. Vornberger, *Chem. Ber.*, **105**, 1084 (1972)]. The salt-free nature of the ylide was in each case confirmed by ^{13}C NMR spectroscopy. The values found for $J(^{13}C-^{31}P)$ were completely in accordance with literature data (see T. A. Albright and E. E. Schweizer, *J. Org. Chem.*, **41**, 1168 (1976); K. A. Ostoja Starzewski and M. Feigl, *J. Organomet. Chem.*, **93**, C-20 (1975); and ref 34b).
(35) W. Schneider and H. F. W. Meyer, *Chem. Ber.*, **54**, 1500 (1921).
(36) Assuming pseudo-first-order kinetics (excess ylide) for the ester conversion.

Reaction of a Mixed Anhydride with Aqueous Hydroxylamine. A Model for the Trapping by Added Nucleophiles of Anhydride Intermediates in Carboxypeptidase A Action

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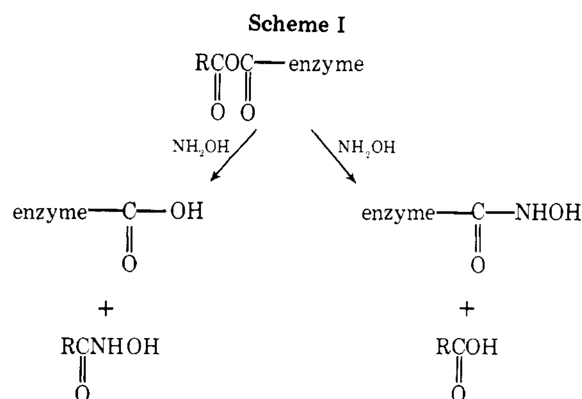
As a model for experiments on the trapping by nucleophiles of acyl-enzyme intermediates formed in the action of carboxypeptidase A, the reaction of *trans-p*-chlorocinnamic propionic anhydride with aqueous hydroxylamine has been examined. Both above and below the pK_a of hydroxylamine, formation of propionohydroxamic acid was found to occur in very high yields. The other dominant product was *trans-p*-chlorocinnamic acid. The pH–rate constant profile for the attack of hydroxylamine on the mixed anhydride was sigmoidal, with an apparent pK_a value of 6.07 ± 0.11 and a limiting second-order rate constant of 2340 $M^{-1}\ s^{-1}$ calculated in alkaline solution. Within the limits of our measurement, catalysis of anhydride breakdown occurred only with the unprotonated form of hydroxylamine. The results obtained suggest that if the acyl-enzyme intermediate observed in kinetic measurements on the reaction of carboxypeptidase A with *O*-(*trans-p*-chlorocinnamoyl)-L-β-phenyllactate is an anhydride species, nucleophilic trapping with hydroxylamine in the absence of interaction of the active site metal ion with the anhydride may be accomplished in reasonable yields.

The trapping of acyl-enzyme intermediates by the use of potent nucleophiles is a useful mthod which has aided in the elucidation of the structure of reaction intermediates involved in enzymic hydrolysis reactions. Trapping experiments in which hydroxylamine was employed as the nucleophile have been carried out on a variety of peptidases including chymotrypsin,[1] pepsin,[2] and bacterial carboxypeptidase.[3] In many of the trapping experiments performed, hydroxylamine was found to be incorporated into the substrate, giving rise to the formation of a hydroxamic acid derived from the substrate. The enzymes involved have generally been ones which form acyl-enzyme intermediates using serine hydroxyl groups at the active site.

Recently, in a study of the reaction of *O*-(*trans-p*-chlorocinnamoyl)-L-β-phenyllactate with carboxypeptidase A at low temperature in a mixed organic–aqueous solution, kinetic evidence was obtained for the intermediate formation of an acyl-enzyme species.[4] On the grounds that the attacking nucleophile at the enzyme active site was probably the γ-carboxylate group of Glu-270, it was proposed that the acyl-enzyme intermediate had a mixed anhydride structure. In order to test such a hypothesis, nucleophile trapping experiments have been undertaken in our laboratory on the reactions of carboxypeptidase A with ester substrates. Some evidence exists that in cases where zinc ion catalyzes the breakdown of model mixed anhydrides, it is not feasible to use hydroxylamine as a trapping nucleophile,[5] and there are a considerable number of trapping experiments which have been carried out on the native zinc-containing enzyme without any success.[6,7] Therefore, the trapping experiments which we are currently performing on the proposed mixed anhydride species formed at the active site of carboxypeptidase are being done in such a way that the trapping is carried out under conditions where the active site metal ion is coordinated to a strongly bound complexing agent. If the metal ion is prevented from interacting with the mixed anhydride formed at the active site of carboxypeptidase, then a reasonable model for the trapping process in the case of the *trans-p*-chlorocinnamoyl-enzyme should be a study of the reaction of a mixed anhydride such as *trans-p*-chlorocinnamic propionic anhydride (I) with aqueous hydroxylamine. If trapping of the acyl-enzyme with hydroxylamine were successful, then either of the two pathways illustrated in Scheme I might be observed. According to one pathway, hydroxylamine would attack the carbonyl group derived from the active site carboxyl of the enzyme and would result in the formation of an enzyme-bound hydroxamate species. The other pathway would involve attack of the nucleophile on the substrate-derived carbonyl group. Similarly, as illustrated in Scheme II, attack at either the *trans-p*-chlorocinnamoyl group or the propionyl group of I might be observed in the reaction of aqueous hydroxylamine with this model mixed anhydride. In the present article we have described the results we have obtained both on the kinetics of reaction of hydroxylamine with I in aqueous solution and on the product distribution, and we have discussed the

Scheme I



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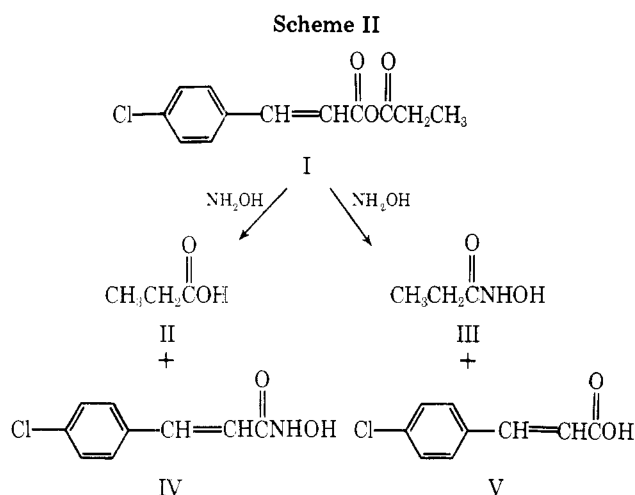
Scheme II



implications of these results for the trapping experiments being performed on reactions of carboxypeptidase A.

Results and Discussion

Kinetic Experiments on the Reaction of I with Aqueous Hydroxylamine. Kinetic experiments on the reaction of I with hydroxylamine were carried out in the presence of the nucleophile in excess. Under these conditions, the kinetics observed were pseudo-first-order in nature. Pseudo-first-order rate constants were obtained by recording the decrease in the UV absorption at 310 nm due to the disappearance of I. Plots of the rate constants measured vs. the hydroxylamine concentrations gave straight lines. From the slopes of these plots, second-order rate constants measured over a range of pH were calculated. In Table I data on the pH dependency of the second-order rate constants are illustrated. The pH–rate constant profile seen was sigmoidal and a pK value of 6.07 ± 0.11 was measured with a limiting second-order rate constant of 2340 $M^{-1} s^{-1}$ calculated in alkaline solution. The rate data obtained clearly indicated that it is the unprotonated form of hydroxylamine which is the reactive species.

Product Distribution. As discussed in the introduction two pathways are possible for the reaction of I with hydroxylamine; one pathway gives propionic acid (II) and *trans-p*-chlorocinnamohydroxamic acid (IV) while the other results in the formation of propionohydroxamic acid (III) and *trans-p*-chlorocinnamic acid (V) (Scheme II). In order to determine the degree to which each pathway is followed, the yields of the hydroxamic acids III and IV were determined. After chromatographic separation, quantitative analysis of the concentrations of the hydroxamic acids produced was carried out colorimetrically by means of the ferric ion–hydroxamate complexes,[8] as summarized in Table II. The results shown in the table indicate that formation of III predominates over that of IV over a wide pH range both above and below the pK_a of hydroxylamine. The gradually decreasing total yield of the hydroxamic acids formed as the pH was raised might be due to the increasingly competitive rate of the spontaneous hydrolysis of I under the reaction conditions.

Analysis by high-pressure liquid chromatography revealed that *trans-p*-chlorocinnamic acid (V) was obtained as a major product in the reaction of hydroxylamine with I. This result is what would be expected in view of the results just discussed for hydroxamic acid production. In Table III the relative yields of IV and V calculated from the peak areas for these compounds determined by high-pressure liquid chromatography are shown. If spontaneous hydrolyses were negligible relative to nucleophilic attack by hydroxylamine, *trans-p*-chlorocinnamic acid (V) should be produced in amounts identical to that of propionohydroxamic acid III. A slightly higher yield of V was obtained than would be expected from

Table I. Second-Order Rate Constants for Reaction of I with Aqueous Hydroxylamine at 25 °C

pH	buffer[a]	k_2, M^{-1} s^{-1} [b]	r^2 [c]
4.0	acetate	37.8	0.994
4.5	acetate	90.4	0.981
5.0	acetate	3.12×10^2	0.989
5.5	cacodylate	5.51×10^2	0.985
6.0	cacodylate	1.35×10^3	0.997
6.5	cacodylate	1.39×10^3	0.971
7.0	cacodylate	2.10×10^3	0.997
7.5	cacodylate	2.02×10^3	0.992
8.0	Tris	2.37×10^3	0.993
9.0	Tris	2.46×10^3	0.989
10.0	ammediol	2.47×10^3	0.987

[a] 9.1% (v/v) of tetrahydrofuran. Buffer concentration was 0.05 M. [b] Data were calculated from at least six points. [c] Correlation coefficients from the plots of the pseudo-first-order rate constants vs. hydroxylamine concentrations.

the product analysis for the hydroxamic acids III and IV, and this may be due to the competing spontaneous hydrolysis of I.

The predominant formation of propionohydroxamic acid (III) and *trans-p*-chlorocinnamic acid (V) indicates clearly that hydroxylamine attacks predominantly the aliphatic carbonyl group of I. One explanation of this observation is that the carbonyl group of the *trans-p*-chlorocinnamoyl moiety in I is conjugated with a double bond; thus, the electron deficiency on the carbonyl carbon is much less than on that of the propionyl moiety. In line with our findings, the exclusive addition of isotopically labeled water to the aliphatic carbonyl group in a mixed aliphatic aromatic carboxylic acid anhydride has been reported by Bunton and Perry.[9]

In summary, the reaction of hydroxylamine with the mixed anhydride I occurred mainly at the carbonyl group of the saturated acid moiety, probably due to the greater electron deficiency of that carbonyl function. The present findings suggest that the attack of hydroxylamine on a mixed anhydride formed from the reaction of *O*-(*trans-p*-chlorocinnamoyl)-L-β-phenyllactate with carboxypeptidase A should result in the incorporation of the hydroxylamine in the enzyme, presumably at the Glu-270 residue. This expectation is consistent with preliminary observations in our laboratory. The tendency for hydroxylamine to attack the carbonyl group of the enzyme-bound residue is in contrast to the situation observed for hydrolysis reactions catalyzed by chymotrypsin in which hydroxylamine was found to form hydroxamic acids derived from the substrate molecules.[1] Thus, the observations described in this paper, together with preliminary data on carboxypeptidase A, show that the failure of a trapping nucleophile to attack the substrate carbonyl group does not ncessarily exclude the existance of an acyl-enzyme intermediate. We are continuing to explore the incorporation of hydroxylamine in carboxypeptidases in their reactions with a number of different substrates.

Experimental Section

General. Nuclear magnetic resonance spectra were recorded on a Bruker HS-270 spectrometer at 270 MHz with a Nicolet NIC-1080 data processor system. Both kinetic experiments and product analyses were carried out with freshly prepared I in tetrahydrofuran. High-pressure liquid chromatography analysis was performed with a Perkin-Elmer series 2 liquid chromatograph.

Materials. Sodium *trans-p*-chlorocinnamate prepared from the reaction of sodium hydride with *trans-p*-chlorocinnamic acid (Aldrich) was recrystallized from aqueous ethanol. Propionyl chloride was distilled under nitrogen (bp 78–79 °C). Tetrahydrofuran was distilled from lithium aluminum hydride under nitrogen. Hydroxylamine hydrochloride (Fisher) was recrystallized from methanol. Other

Table II. Yields of Hydroxamic Acids for Reaction of I with Aqueous Hydroxylamine

pH	buffer[b]	hydroxylamine concentration, M	yields,[a] (%) III	IV	total
5.0	acetate	1.82×10^{-2}	78.7 (95.5)	3.7 (4.5)	82.4
6.0	cacodylate	1.95×10^{-2}	78.3 (95.0)	4.1 (5.0)	82.4
7.0	cacodylate	1.98×10^{-2}	71.2 (94.9)	3.8 (5.1)	75.0
8.0	Tris	1.84×10^{-2}	58.5 (95.3)	2.9 (4.7)	61.4

[a] Yields were calculated on the basis of the amount of propionyl chloride used in the preparation of I, assuming quantitative conversion of the acid chloride into the mixed anhydride. Relative yields of the hydroxamic acid are shown in parentheses. The initial concentration of I was 3.9×10^{-3} M. [b] 9.1% (v/v) tetrahydrofuran was present. The buffer concentration was 0.05 M.

Table III. Relative Yields of IV and V[a]

pH[b]	relative yields, % IV	V
5.0	3.6	96.4
6.0	3.5	96.5
7.0	3.7	96.3

[a] The initial concentrations of I and hydroxylamine were 3.54 $\times 10^{-4}$ and 1.25×10^{-2} M, respectively. [b] The buffer (0.01 M cacodylate) contained 16.7% of tetrahydrofuran.

chemicals were of C.P. grade and were used without further purification. Microanalysis was performed by Microtech Laboratories.

Preparation of Tetrahydrofuran Solution of I. A suspension of 250 mg (1.22 mmol) of sodium *trans-p*-chlorocinnamate in 5 mL of tetrahydrofuran was mixed with 40 μL (0.46 mmol) of propionyl chloride at 0 °C under nitrogen with stirring. After stirring for 1 h, the reaction mixture was centrifuged at 6000 rpm (Beckman JA-21B centrifuge with JA-20 rotor) for 15 min at 0 °C. The supernatant was collected. The precipitate was resuspended in 2 mL of cold tetrahydrofuran and centrifuged at 6000 rpm for 10 min at 0 °C. The combined supernatant was diluted in a 10-mL volumetric flask with tetrahydrofuran. A sample for NMR spectroscopy was prepared by replacing the solvent by dimethyl-d_6 sulfoxide. The NMR spectrum showed the following peaks (ppm from Me_4Si): 1.10 (3 H, triplet, J = 7.37 Hz), 2.63 (2 H, quartet, J = 7.37 Hz), 6.78 (1 H, doublet, J = 16.1 Hz), 7.53 (2 H doublet, J = 8.5 Hz), 7.83–7.85 (3 H, overlap of two doublets, J = 8.3 and 16.5 Hz). In a freshly prepared solution of I less than 1% *trans-p*-chlorocinnamic anhydride was found. The completion of the consumption of the propionyl chloride used in the preparation was checked by argentometric titration. One milliliter of the tetrahydrofuran solution of I prepared as described above was poured into 5 mL of deionized water. After 5 min of standing at room temperature, 5% potassium chromate was added and the resultant solution was titrated with standardized silver nitrate solution. No difference was found between the hydrolyzed sample and an appropriate blank sample.

Because on concentration I was found to isomerize readily to the symmetrical anhydride, no further attempt was made to isolate I. In tetrahydrofuran I was found to be stable at concentrations below 10^{-3} M for a couple of weeks at 4 °C.

Preparation of Hydroxamic Acid. Propionohydroxamic acid was prepared as described in the literature,[10] mp 93.5–94.5 °C (lit.[11] mp 92.5–93.0 °C). *trans-p*-Chlorocinnamohydroxamic acid was prepared by the reaction of *trans-p*-chlorocinnamoyl chloride with hydroxylamine.[7] Anal. Calcd for $C_9H_8ClNO_2$: C, 54.70; H, 4.08; N, 7.09. Found: C, 54.53; H, 4.15; N, 7.02.

Kinetic Experiments. Kinetic runs were carried out using a Durrum-Gibson stopped-flow spectrophotometer with a 20 mm flow cell. One volume of the tetrahydrofuran solution of I (9.2×10^{-5} M) was mixed with ten volumes of hydroxylamine solution at 25.0 °C. The reaction corresponding to the disappearance of compound I was monitored at 310 nm. The hydroxylamine solution was prepared by dissolving hydroxylamine hydrochloride in the appropriate buffer and adjusting the resultant solution to the pH desired by the addition of 1 N sodium hydroxide.

Analysis of Hydroxamic Acid Products. One milliliter of a solution of I in tetrahydrofuran (4.3×10^{-2} M based on the amount of propionyl chloride used in the preparation) was mixed with 10 mL of hydroxylamine solution (the concentrations of hydroxylamine and the buffers employed are shown in Table II) at 25 °C. Hydroxylamine was purified in the same manner as described in the section where the kinetic experiments were discussed. After at least 10 min, an aliquot (1 or 2 mL) was applied to a DEAE–cellulose column equilibrated with 0.01 M Tris–HCl, pH 7.5 (void volume 8 mL). In the cases of the samples at pH 5.0 and 6.0, 0.1 mL of 1 N sodium hydroxide was added to the reaction mixture to dissolve the white precipitate which appeared. The column was connected to a Technicon Auto Analyzer (Model NC-1). Elution with 0.01 M Tris–HCl buffer at pH 7.5 was carried out for the first 15 min, followed by 0.1 M Tris–HCl at pH 7.5 at a flow rate of 1.2 mL/min. The effluent from the column was mixed with 5% $FeCl_3 \cdot 6H_2O$ in 1 N HCl at an equal flow rate. The hydroxamic acids were then detected by the use of a 1 cm flow cell equipped colorimeter (filter 570-18-28). Compound III was eluted with 0.01 M Tris–HCl at 10 min and IV appeared at 40 min after the eluent was changed to 0.1 M Tris–HCl. The yields of hydroxamic acid obtained were calculated from the peak areas. Calibration of the analytic methodology employed was performed using authentic samples.

Analysis for *trans-p*-Chlorocinnamic Acid Produced. The reaction of I with hydroxylamine in which the yield of *trans-p*-chlorocinnamic acid was determined was performed in a manner similar to that described above, except that 0.5 mL of I in tetrahydrofuran was mixed with 3.5 mL of hydroxylamine containing buffered solution. Analysis for formation of the *trans-p*-chlorocinnamic acid was carried out by high-pressure liquid chromatography using an ODC–silica gel reversed phase column and an eluent consisting of 30% acetonitrile–70% aqueous phosphate (v/v), pH 3.8. The hydroxamic acid, IV, and the carboxylic acid, V, were detected at 280 nm where the molar extinction coefficients employed were 2.7×10^4 and 2.48×10^4, respectively.

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Registry No.—I, 66793-00-8; III, 2580-63-4; IV, 29900-76-3; V, 940-62-5; V sodium salt, 66793-01-9; propionyl chloride, 79-03-8; hydroxylamine, 7803-49-8.

References and Notes

(1) (a) R. M. Epand and I. B. Wilson, *J. Biol. Chem.*, **238**, 1718 (1963); (b) T. Inagami and J. M. Sturtevant, *Biochem. Biophys. Res. Commun.*, **14**, 69 (1964).
(2) Y. Nakagawa, L.-H. King Sun, and E. T. Kaiser, *J. Am. Chem. Soc.*, **98**, 1616 (1976).
(3) T. Nishino, J. W. Kozarich, and J. L. Strominger, *J. Biol. Chem.*, **252**, 2934 (1977).
(4) M. W. Makinen, K. Yamamura, and E. T. Kaiser, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 3882 (1976).
(5) R. Breslow, D. E. McClure, R. S. Brown, and J. Eisenach, *J. Am. Chem. Soc.*, **97**, 194 (1975).
(6) R. Breslow and D. L. Wernick, *PROC/ Natl. Acad. Sci. U.S.A.*, **74**, 1303 (1977).
(7) J. Suh, Ph.D. Thesis, University of Chicago, 1975.
(8) L.-H. King and E. T. Kaiser, *J. Am. Chem. Soc.*, **96**, 1410 (1974). See also: F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **159**, 21 (1945).
(9) C. A. Bunton and S. G. Perry, *J. Chem. Soc.*, 3070 (1960).
(10) A. Miolati, *Ber.*, **25**, 699 (1892).
(11) L. W. Jones and L. Neuffer, *J. Am. Chem. Soc.*, **39**, 659 (1917).