RNase-all D at 49° have been determined by the technique described above, and will be reported in the near future.

Acknowledgment.—We are indebted to Dr. W. D. Cooke for calling our attention to the usefulness of the overtone region.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

The α-Chymotrypsin Catalyzed Hydrolysis of *p*-Nitrophenyl Acetate[1a]

BY MARIO A. MARINI[1b] AND GEORGE P. HESS

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By the criteria of kinetic, chemical and spectroscopic studies of the α-chymotrypsin catalyzed hydrolysis of *p*-nitrophenyl acetate, there appears to be at least two stable monoacetyl-α-chymotrypsin intermediates, AC-A and AC-I, in the catalytic reaction. AC-I is kinetically identical to the normal acyl intermediate at *p*H >6.2. At *p*H 5.0, AC-I can rearrange to give AC-A which is identical to the monoacetyl-α-chymotrypsin isolated by Balls and Wood but kinetically, chemically and spectroscopically different from AC-I. Experiments on the chemical reactivity of AC-A suggest that it is homogenous. On the assumption that the aliphatic hydroxyl group of serine and the imidazoyl group of a histidine residue participates in the catalytic reaction, a structure for the active site of AC-A is proposed which would account for all the data presented here.

The kinetics of the α-chymotrypsin catalyzed hydrolysis of *p*-nitrophenyl acetate has been interpreted by Gutfreund and Sturtevant in terms of three catalytic steps.[2] After the formation of an enzyme–substrate complex, a monoacyl enzyme capable of isolation[3] is formed, follwed by the decomposition or hydrolysis of this complex. The hydrolysis of specific substrates for α-chymotrypsin probably involves the same three step mechanism as the hydrolysis of *p*-nitrophenyl acetate.[4,5]

Many investigators have sought the nature of the group or groups involved in the catalytic reaction. Both an aliphatic hydroxyl group of serine[6] and an unprotonated imidazoyl group of a histidine[7] residue have been implicated. The evidence that serine is present[8,9] in a single active site[10] is fairly clear. The evidence for histidine is largely indirect[11,12] and of a more controversial nature.[13,14]

Direct nucleophilic attack by an unprotonated imidazoyl nitrogen on the substrate might form the N-acylimidazoyl derivative[15] on the enzyme surface. Evidence for this view is derived, in part, from studies of the phosphorylated derivatives of chymotrypsin.[16] The phosphorylated chymotrypsin is dephosphorylated with faster rates under acidic conditions which is easily explained if imidazole is the phosphorylated group. Objections to this mechanism have been raised. Dixon and Neurath[17] were unable to detect a species absorbing at 245 mμ, characteristic for N-acetyl imidazole,[18] during the formation of the monoacetyl enzyme in the reaction of chymotrypsin with *p*-nitrophenyl acetate at low *p*H. Cunningham[19] also objected to this mechanism on the basis that the heat of ionization of the rate determining group in the acylation of chymotrypsin is 11 kcal. mole^{-1},[20] while only 6–7 kcal. would be expected if a direct attack of a normally ionizing imidazoyl nitrogen were involved. He proposes an alternate mechanism which proceeds by nucleophilic attack of a serine hydroxyl group which is activated by hydrogen bonding to imidazole. Hydrolysis of the acyl-enzyme occurs by attack of another nucleophilic reagent. This nucleophilic reagent may be the imidazoyl group of histidine which would then form an N-acyl imidazole and then hydrolyze rapidly in water.[21,22]

Dixon and Neurath,[21] from their spectroscopic studies, found that the isolated monoacetyl chymotrypsin was deacylated at high *p*H with an accompanying increase in absorption at 245 mμ and noted that the absorption maximum and the rate of de-

(1) (a) This work was aided by grants from the United States Public Health Service and the National Science Foundation.(b) Post-doctoral Fellow, National Heart Institute, United States Public Health Service.

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crease of the absorbancy are suggestive of N-acetyl imidazole.[18] From their data, they suggest an initial attack on the substrate by a serine hydroxyl group and a subsequent shift of the acyl group to imidazole which is followed by the base catalyzed hydrolysis of the N-acylimidazoyl derivative. This mechanism also presents serious problems. If the rate of decay of the 245 mμ peak is the decay of N-acetyl imidazole, it has a rate constant of 1.7×10^{-3} sec.$^{-1}$ while the rate limiting step for the hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin is more than 10 times as fast (2.5×10^{-2} sec.$^{-1}$)[2] under the same conditions. Also when α-chymotrypsin and *p*-nitrophenyl acetate are reacted at *p*H 6.6, the enzyme takes up hydrogen ions from the buffer.[2] If the imidazole (*pK* 6.7) were acetylated, its *pK* would be much lower (calculated *pK* 3.6)[23] and under these conditions should release protons and not take them up.

Many of the discrepancies would be explained if the various preparations of the isolated intermediate (monoacetyl-chymotrypsin) differed from each other or from the true intermediate. Spencer and Sturtevant[4] presented evidence that the isolated monoacetyl-α-chymotrypsin of Balls and Wood[3] was the true intermediate and that for both acetyl-L-tyrosine ethyl ester and *p*-nitrophenyl acetate, the same mechanism was applicable. Preliminary reports[24-26] by the present authors indicated that the isolated material was kinetically and chemically not identical to the monoacetyl enzyme formed during the normal catalytic hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin at *p*H > 6.2.

This paper reports the methods used and the data obtained to differentiate the true catalytic intermediate from the isolated monoacetyl-α-chymotrypsin. An understanding of the difference would give an insight to the groups involved in the active site and might suggest a mechanism correlating the data thus far obtained with the various preparations used.

Results and Discussion

Kinetic Experiments.—The hydrolysis of *p*-nitrophenyl acetate by chymotrypsin and the monoacetyl-α-chymotrypsins (AC-I and AC-A) is plotted in Fig. 1. If chymotrypsin normally catalyzed the reaction by way of monoacetyl-α-chymotrypsin, the initial rate of release of *p*-nitrophenol by monoacetyl-α-chymotrypsin must be identical to the steady state liberation of *p*-nitrophenol by α-chymotrypsin. Since α-chymotrypsin and AC-I are kinetically identical, the catalysis proceeds through some fully acetylated form. Since AC-A, fully acetylated, is not kinetically identical to α-chymotrypsin or AC-I, it must represent a different form of the normal intermediate. This form is capable of returning to the normal catalytic path, as determined by the release of *p*-nitrophenol, for after 300 seconds the rates of *p*-nitrophenol liberation for all the enzymes are identical. This change cannot be ascribed to the isolation procedure be-

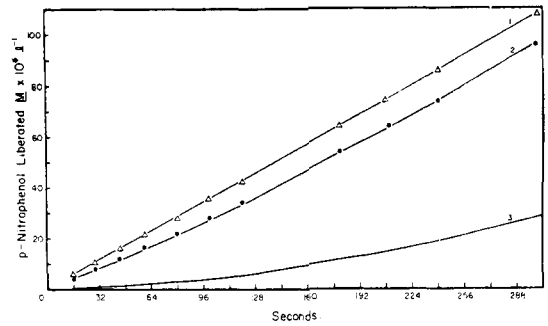

Fig. 1.—Optical density changes in the catalytic hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin and monoacetyl-α-chymotrypsin at 15.6° in tris-(hydroxymethyl)-aminomethane-maleate buffer, *p*H 7.0. Total ionic strength 0.12 *M*; $(E)_0 = 1.6 \times 10^{-5}$ *M*, $(S)_0 = 1.7 \times 10^{-3}$ *M*, 5.0% (v./v.) acetone. Curve 1, chymotrypsin and *p*-nitrophenyl acetate at *p*H 5.0 then mixed with buffer to give *p*H 7.0; and isolated AC-I under the same conditions or when isolated AC-I is added directly to the substrate at *p*H 7.0. Curve 2, AC-A isolated and chymotrypsin preacetylated with 100 equivalents of substrate for 4 hr. at *p*H 5.0 and 15.6° then brought to *p*H 7.0. Curve 3, theoretical curve calculated for monoacetyl-α-chymotrypsin if the acetyl group were decaying at the rate of N-acetyl imidazole decomposition.

cause AC-I and AC-A are treated similarly after acetylation and, in addition, the same slow release of *p*-nitrophenol was seen (Fig. 1, curve 2) when α-chymotrypsin was preacetylated with *p*-nitrophenyl acetate for 4 hours and run without isolation.

The only difference between AC-A, AC-I and the abnormal unisolated intermediate (curve 2) is the length of time which chymotrypsin was allowed to react with *p*-nitrophenyl acetate. Since both AC-I and AC-A are equally acetylated the difference in rate of liberation of *p*-nitrophenol cannot be attributed to a pre-steady state liberation of the acetyl group. Similarly when the rates of deacylation of AC-I and AC-A were measured directly by the hydrolysis of acetyl-L-tyrosine ethyl ester by the free enzyme, using the conditions published previously,[27] AC-I and AC-A again are kinetically different, AC-A being deacylated at a slower rate than AC-I.[28] Some change or rearrangement must have occurred which is the cause of the initial different slow liberation of *p*-nitrophenol from *p*-nitrophenyl acetate by AC-A. Figure 1, curve 3 shows the theoretical curve calculated for monoacetyl-α-chymotrypsin if all the acetyl group migrated to imidazole and deacylated at a rate characteristic of N-acetyl imidazole ($k = 1.7 \times 10^{-3}$ sec.$^{-1}$ at *p*H 8.0 and 20°). The early rate of the reaction (to 30 seconds) of the theoretical curve is only about 2% of the final rate but the early rate (15–30 seconds) of AC-A (curve 2) is 70% of the final rate. It would seem that only 30% of AC-A could deacylate via N-acetyl imidazole, if at all. Since Dixon and Neurath[21] used the same AC-A[3] to obtain evidence for the formation of acetyl-imidazole, perhaps this is the anomalous form. They detected 0.4 equivalents of the N-acetyl derivative which could repre-

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sent all of the acyl group migrated. This would be in agreement with the 0.3 equivalent suggested by the present study.

It is apparent that AC-A deacylates slower than the normal intermediates, α-chymotrypsin and AC-I. It also deacylates faster than it would if the deacylation proceeds entirely *via* N-acetyl imidazole. If it deacylates via N-acetyl imidazole at all, the kinetic experiments indicate that only 0.3–0.4 equivalents of the acetyl group could migrate to the imidazole nitrogen. About 0.7 equivalents of the acetyl group presumably proceeds through the normal mechanism. This can be explained by two alternatives: (a) AC-A consists of 70% normal intermediate and 30% of a form deacylating *via* N-acetyl imidazole or (b) AC-A is a single homogenous intermediate which under the hydrolytic conditions employed gives rise to two separate intermediates, 30% of which is the N-acetyl imidazole derivative.

Hydroxylamine Reaction.—All hydroxamate tests were conducted on various samples of α-chymotrypsin or its derivatives and run in triplicate. Results from any given day showed little variation, but the daily reproducibility was poor. This was found to be a fault of the blank used. Reagent blanks in water and urea for the standards were good but the test blanks using chymotrypsin were subject to wide variation in triplicate runs. Use of the inverted blank, that is, addition of chymotrypsin after the addition of acid in the hydroxamate test, gave values lower than the reagent blank; the monoacetyl-α-chymotrypsins, AC-A or AC-I, deacylated for 20 minutes at *p*H 8.0 gave high values which may reflect the incomplete deacylation under these conditions. Chymotrypsin which was subjected to the isolation procedure used for monoacetyl-α-chymotrypsin, but without acetylation, was ultimately used as the blank. The degree of acetylation of AC-A and AC-I determined using this blank, gave consistently low values, although all acetylated preparations used were completely acetylated when the initial burst of *p*-nitrophenol was used as the criterion.[29]

The reaction of hydroxylamine with AC-I and AC-A indicates a difference in reactivity of the acetyl group in these preparations and provides further evidence that these two intermediates are not identical. Hydroxylamine is known to react at low *p*H with "activated" esters[3,30,33] while being generally unreactive toward the normal esters. Monoacetyl-α-chymotrypsin, in water, will react with hydroxylamine[3] while O-acetyl serine would be unreactive at low *p*H.[31] If the acetyl group is attached to serine in the native enzyme, it would not react unless it were activated by some nucleophilic side chain, presumably histidine. Sequence studies have not located histidine[15,32] in the chain with serine, therefore, it is presumably present in the site by means of folding of the peptide chain and can be removed from the serine site by denaturing the enzyme with urea. Once denatured, the *O*-acetyl serine would then be unreactive toward hydroxylamine at low *p*H. This was borne out by the studies of Dixon, *et al.*,[33] and again in Table I. Monoacetyl-α-chymotrypsin in water gives a positive hydroxamate, but after being allowed to stand for 2 minutes at 23° in 8 *M* urea at *p*H 5.5, it gives no test. This is not due to the hydrolysis of the acetyl group from the enzyme, for the denatured enzyme gives a fully positive hydroxamate reaction at *p*H 11 showing that hydrolysis of the acetyl group had not occurred. The anomolous intermediate, AC-A, gives a positive test both in water and after denaturation under the same conditions. Longer treatment with urea at *p*H 5.5 (Table II) also abolishes the reactivity of AC-A toward hydroxylamine. The activity toward hydroxylamine of AC-I and AC-A are equally abolished when denaturation was performed at *p*H 3.0, for 2 minutes at 23°.

TABLE I

THE REACTIVITY OF THE MONOACETYL-α-CHYMOTRYPSINS WITH 1 *M* HYDROXYLAMINE

	*p*H			
	3 or 5.5 H_2O	3.0 8 *M* Urea	5.5 8 *M* Urea	11.0 8 *M* Urea or H_2O
AC–I	100[a]	0	5 ± 2	100
AC–A	100[b]	1	79 ± 10	100

[a] The average value for AC–I using a treated chymotrypsin blank was 63 ± 13% which was arbitrarily set to 100. [b] Average value for AC-A is 64 ± 10% from 60 samples of various preparations isolated and unisolated, run in triplicate. (See text.)

TABLE II

THE HYDROXAMATES OF AC–A AFTER VARIOUS TIMES OF DENATURATION

Time, min.	0	0.5	1	2	4	5	10
AC–A[a]	100	84	82	74	46	41	4

[a] Average of six determinations on the isolated material.

Clearly the reactivity of the acetyl groups of AC-A and AC-I toward hydroxylamine differs under these conditions. The hydroxylamine experiments are also compatible with AC-A representing a single intermediate.

Denaturation Experiments.—The rate of denaturation of chymotypsin and AC-A as measured by optical rotation (Fig. 2) shows that they are denatured equally at equal rates. In 2 minutes, both are approximately 90% denatured by this criterion. Assays of the enzymatic activity of chymotrypsin show the same rate of denaturation (Fig. 3) by 8 *M* urea. In 2 minutes, the active site of chymotrypsin is made inactive by removing one of the active components, but in AC-A the site is still active by the criterion of the hydroxylamine reaction while extensive protein denaturation must have occurred. Examination of the data obtained by hydroxamate and denaturation studies gives some insight into the nature of the intermediate AC-A. The active site of AC-A appears to be stabilized by some linkage which urea will eventually cleave, directly or indirectly.

(29) We wish to express our thanks to Dr. Lyman C. Craig for the use of his instrument.

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(33) G. H. Dixon, W. J. Dreyer and H. Neurath, *ibid.*, **78**, 4810 (1956).

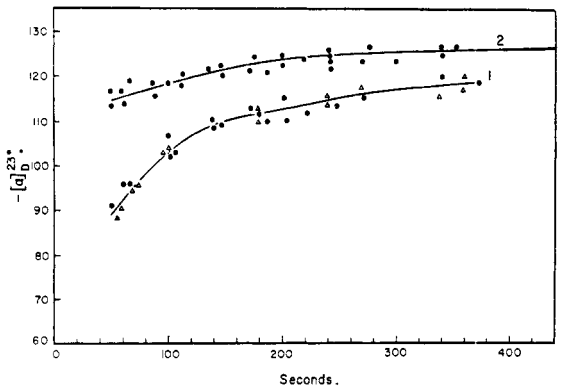


Fig. 2.—Curve 1, the time dependent change in the specific rotation of α-chymotrypsin and isolated monoacetyl-α-chymotrypsin, in 8 *M* urea at 23° in 0.05 *M* tris-maleate buffer at *p*H 5.5. Curve 2, α-chymotrypsin in 8 *M* urea at *p*H 3.0 in 0.05 *M* sodium citrate buffer. Optical rotation was measured with a Rudolph Photoelectric Spectro-Polarimeter.[29]

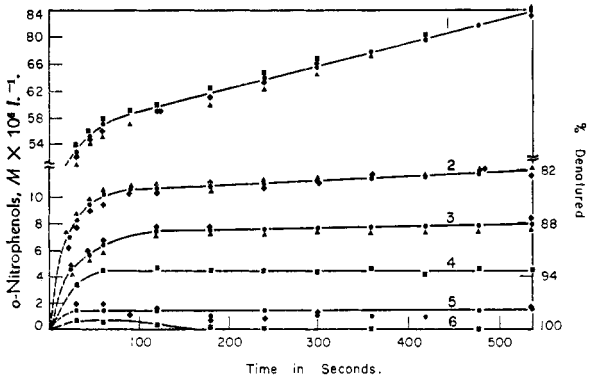


Fig. 3.—The hydrolysis of *p*-nitrophenyl acetate by denatured α-chymotrypsin in 6*M* urea at *p*H 5.5, 18°. $(E)_0 = 5.7 \times 10^{-5}$ *M*, $(S)_0 = 1.7 \times 10^{-3}$ *M*, 5.0% (v./v.) acetone. Curve 1, α-chymotrypsin alone; curve 2, α-chymotrypsin denatured 1 min. in 8 *M* urea; curves 3–5, enzymes denatured for 2, 5 and 10 minutes in 8 *M* urea; curve 6, α-chymotrypsin denatured 2 minutes at *p*H 3.0 in 8 *M* urea.

Spectroscopic Studies.—In the spectroscopic studies, when the intermediates AC-A and AC-I were tested under essentially the same conditions used by Dixon and Neurath[21] and Spencer and Sturtevant[4] only AC-A gave the rise and slow decay at 245 mμ while AC-I was indistinguishable from α-chymotrypsin (Fig. 4). Under the conditions used, none of the preparations gave an initial sharp drop at 245 mμ as reported by Spencer and Sturtevant,[4] and this phenomena is currently being investigated.

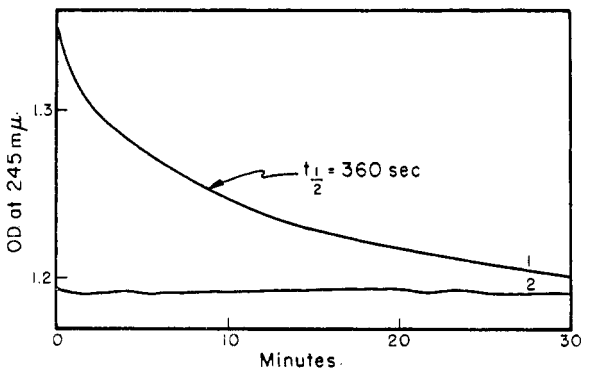
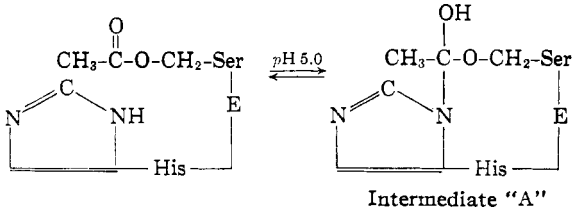


Fig. 4.—The change in optical density at 245 mμ of α-chymotrypsin and the monoacetyl α-chymotrypsins, AC-A and AC-I, in *p*H 8.0 buffer (0.05 *M* tris-maleate, 0.05 *M* $CaCl_2$) at 20°. A Cary, Model 14, selfrecording spectrophotometer was used. Curve 1, AC-A at *p*H 3.0 raised to *p*H 8.0. Curve 2, AC-I or α-chymotrypsin at *p*H 3.0 raised to *p*H 8.0.

Discussion

The data obtained are compatible with AC-A representing a single intermediate. The intermediate must be temporarily resistant to urea denaturation at *p*H 5.5 and more sensitive at *p*H 3.0. It must, on elevating the *p*H, be capable of forming a species absorbing at 245 mμ and decomposing with a rate similar to that of N-acetyl imidazole. Presuming the normal intermediate to be an O-acetyl serine derivative of the enzyme and the anomalous one to be capable of forming an N-acetyl imidazoyl derivative of histidine, a structure can be proposed for the active site of AC-A which would satisfy all the data.



Intermediate "A"

The presence of the proposed Intermediate "A" in proteins, analogous to an oxazolidine, has recently been reviewed.[34] Theoretical considerations predict that *p*H as well as electrostatic interactions have large direct effects on the stability of oxazolidines, as well as on the relative amounts of O-acetyl and N-acetyl derivatives derived from the oxazolidines. The properties of Intermediate "A," as deduced by the experimental findings presented, are in accord with the proposed structure. Because of the covalency of the imidazoyl N to the carboxyl carbon this form could be expected to take longer to disrupt by urea than AC-I in which this covalent bond does not exist. Urea denaturation, in changing the electrostatic interactions, could be expected to rupture this complex. The stability of the complex should furthermore be *p*H dependent and this is again in accord with experimental findings. Intermediate "A" decomposes more rapidly in 8 *M* urea at *p*H 3.0 than at *p*H 5.5 as judged by the hydroxylamine reaction. The relative amounts of O-acetyl and N-acetyl derivatives formed should also be *p*H dependent. The kinetic experiments indicate that the rate of deacylation of AC-A can be best described by two first order rate constants. This may mean that AC-A is deacylated *via* both the O- and the N-acetyl derivatives.

(34) For a review of this subject see: H. A. Saroff, *Enzymologia*, **21**, 101 (1959).

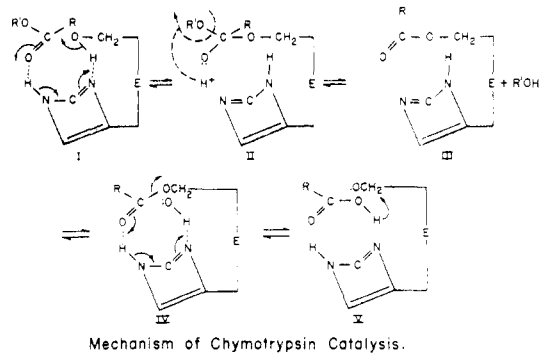
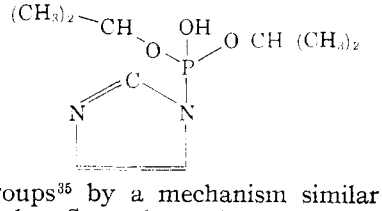


Fig. 5.—Proposed mechanism for the α-chymotrypsin catalyzed hydrolysis of *p*-nitrophenyl acetate.

The postulate that AC-A is in actuality a rearranged product of the normal catalytic mechanism is further borne out by an examination of the inhibited phosphorylated derivatives. It is well known that some esterases may be reactivated if treated with various nucleophilic agents immediately after inhibition.[16] When the phosphorylated intermediate is allowed to stand for a time, it can no longer be reactivated. This time could be necessary to effect the rearrangement to a form analogous to Intermediate "A" followed subsequently by the observed release of one of the iso-



propyl groups[35] by a mechanism similar to that proposed by Samuels and Westheimer.[36] The resulting anion can then resist nucleophilic attack in the regenerating procedures used.

If Intermediate "A" represents the structure of the isolated intermediate, then a mechanism for the normal catalytic reaction may be postulated which would account for both the formation of "A," the nucleophilicity of the serine hydroxyl group and be in accord with the model experiments of poly-functional catalysis[37] (Fig. 5). For the artists convenience, the imidazole is presented here with an invaginated structure while in reality it is planar. Molecular models have been constructed which easily accomodate all the features presented here.

Ester interchange with the serine hydroxyl is promoted by the removal of the hydroxyl hydrogen by the amidine nitrogen and the simultaneous hydrogen bonding with the ester (I). Here the imidazole acts in concerted fashion[37] causing the acylation to proceed rapidly. Such a concerted mechanism would be expected to give much higher rates than a single displacement reaction.[38] The *pK* of the group in the acylation step is 6.7[11,20] while that of imidazole is 7.0.[39] If the serine is hydrogen bonded to the imidazole, the *pK* should fall as is observed. Release of the alcoholate (II) is the result of the interchange which gives the normal O-acetyl serine intermediate (III). Under normal conditions of hydrolysis the imidazole, as a donor, could hydrogen bond to the carbonyl oxygen (IV) explaining the higher *pK* observed[20,27] in the deacylation reaction. The amidine nitrogen then can be hydrogen bonded to water or to an hydroxide ion to increase its nucleophilicity and the subsequent concerted attack would release the acid and regenerate the enzyme.

Hydrogen bonding in (I) and (IV) serve the additional purpose of retaining the carbonyl oxygen of the substrate as a carbonyl since it has been shown[40] that no symmetrical addition of water occurs in chymotrypsin catalysis.

In the proposed mechanism the imidazole nitrogen serves as a specific site for hydroxyl ion. In absence of sufficient OH^- concentration as at *p*H 5.0, the partially unprotonated imidazole nitrogen can now attack the carbonyl carbon of the O-serine ester leading to formation of Intermediate "A." At higher *p*H, the carbonyl group is removed directly from the imidazole nitrogen by the hydroxide ion. The limiting rate of the reaction can still become independent of hydroxide concentration, as is experimentally found, once all the specific sites for OH^- become saturated.

If intermediate (III) leads to Intermediate "A" in the manner shown, then the O to N shift, the hydroxamate data and the unusual behavior with the phosphates can be reasonably explained. Certain objections can be answered but with no great degree of assurance. Spencer and Sturtevant[4] question that (I) is necessary since (IV) essentially reproduces the circumstances needed for acylation of the enzyme. Why then have (I)? In some cases ester interchange may be energetically more favorable than direct hydrolysis[41] and it is conceivable that such a reaction may be utilized at the active site. The hydrolysis of the acyl group from the enzyme can be accomplished by the same group at a slower rate because of the more favorable steric factors after the initial rapid acylation. Since O-acetyl serine is isolated[9] from the reaction of α-chymotrypsin and *p*-nitrophenyl acetate, the monoacyl enzyme is formed and it probably involves serine. Then (I) probably is correct. It is more appropriate to question (IV). The monoacyl enzyme must hydrolyze by some means and the imidazole nitrogen is the logical group to explain the unusual *p*H dependence of the hydrolysis although mechanisms[42,43] have been proposed which avoid the use of histidine.

Other groups can be visualized in the place of imidazole since the proposed mechanism requires

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a nucleophilic and an electrophilic group acting in concerted fashion. The identity of these groups is conjectural at present since histidine is not known unequivocally to be present. Since the true intermediate (AC-I) and the normally isolated intermediate (AC-A) are different, the nature of the groups in the catalytic site must be responsible for the rearrangement of AC-I to AC-A. Current efforts are being directed at the elucidation of the nature of the groups which cause this rearrangement and which must be part of the active site of α-chymotrypsin.

Experimental

Materials.—*p*-Nitrophenyl acetate was prepared from *p*-nitrophenol (recrystallized from 0.001 *M* HCl, m.p. 114–116°) and acetic anhydride and recrystallized from hexane to give light yellow crystals, m.p. 80°.[44] α-Chymotrypsin, 3 times recrystallized and salt-free, was purchased from Worthington Biochemical Co., Freehold, N. J. and used without further purification. All other materials were reagent grade.

Determination of Protein Concentration.—Protein concentrations were determined spectrophotometrically at 280 mμ by using $E_{1}\%^{280} = 20.0$ to relate extinction to protein concentration.[27] The molecular weight of α-chymotrypsin was taken as 23,000.

Monoacetyl α-Chymotrypsin (AC-A).—The isolation procedure of Balls and Wood[3] was used unmodified to prepare the commonly isolated intermediate (AC-A). It was found that lyophilization of this preparation had no effect on the measurements reported in this paper.

Monoacetyl α-Chymotrypsin (AC-I).—This was prepared by allowing the chymotrypsin solution to react with *p*-nitrophenyl acetate for 10 minutes at *p*H 5.0, as above, at which time only one equivalent of the substrate had reacted as determined spectrophotometrically by the amount of *p*-nitrophenol liberated. Monoacetyl α-chymotrypsin (AC-I) prepared in this way could be isolated by the normal procedure if done rapidly in the cold and the values obtained with the lyophilized material are identical with those obtained with the unisolated preparations.

Determination of Extent of Acylation.—Complete acetylation of the reactive group of α-chymotrypsin was ascertained both by spectrophotometric methods[27] and by reaction with hydroxylamine.

Hydroxamates.—The procedure of Hestrin[45] was modified as follows: one ml. of the monoacetyl-α-chymotrypsin (0.5–1.0 μ*M*) or the standard (0.8 μ*M*/ml. *p*-nitrophenyl acetate in water) was added to 4 ml. of 0.05 *M* tris-maleate buffer (*p*H 5.5) and allowed to stand 2 minutes at 18°. The buffer was prepared in water or in 10 *M* urea. To this solution was added 1 ml. of freshly prepared 4 *M* hydroxylamine *p*H 5.5, mixed and allowed to stand 10 minutes. The reaction was stopped by the addition of 2 ml. of hydrochloric acid of a concentration such that the final *p*H was 1.0 ± 0.1 as predetermined by the glass electrode. The proteins were incompletely precipitated by trichloroacetic acid so 1 ml. of 10% sodium tungstate was found to be more advantageous for this step. The color was developed with 5 ml. of 5% ferric chloride in 0.1 *N* hydrochloric acid, and all tubes were filtered into 25 mm. matched tubes and read at 540 mμ in a B & L spectrophotometer. Each determination was run on triplicate samples. Reagent blanks for the standards used water in place of the ester. The blanks for monoacetyl-α-chymotrypsin were: (a) chymotrypsin treated as indicated omitting the acetylation with the substrate, (b) monoacetyl-α-chymotrypsin deacylated 20 minutes at *p*H 8.0 and (c) chymotrypsin which was added prior to the precipitation with tungstate.

Kinetics.—One ml. of α-chymotrypsin or derivative at *p*H 5.0 and 1 ml. of *p*-nitrophenyl acetate were mixed in a 3 ml. Beckman cuvette and placed in a thermostated Beckman cell holder at 15.5 ± 0.2 for 2 minutes. The enzyme concentration was usually 8.7×10^{-5} *M*. At zero time, 1 ml. tris-maleate buffer (0.05 *M*) at the appropriate *p*H was added and the change in absorption at 410 mμ recorded with time. In the case of α-chymotrypsin this procedure allows the preacetylation (*i.e.* "burst")[46] to occur prior to the actual hydrolysis resulting in the measurement of the steady state liberation of *p*-nitrophenol. The experimental points reported represent the average of 15 experiments run in triplicate.

Determination of Extent of Denaturation.—(a) Enzymatic: Since α-chymotrypsin was denatured at *p*H 5.5, advantage was taken of its ability to react with one equivalent of *p*-nitrophenyl acetate at this *p*H. Only the undenatured form of chymotrypsin will acetylate and the amount of *p*-nitrophenol liberated is a direct measure of the remaining amount of native chymotrypsin. The denaturation was accomplished by adding 1 ml. of α-chymotrypsin to 1 ml. of 16 *M* urea at *p*H 5.5 (tris-maleate 0.05 *M*) in a cuvette in a thermostated (18°) Beckman compartment. After 2 minutes, 1 ml. of this solution was mixed with 2 ml. of *p*-nitrophenyl acetate (10 mμ) in 5.2 *M* urea at *p*H 5.5 (tris-maleate 0.05 *M*) and the absorption at 330 mμ[47] at zero time was taken as the amount of undenatured chymotrypsin. An alternate procedure for determining the amount of native α-chymotrypsin remaining after urea treatment was the use of the substrate tyrosine ethyl ester at *p*H 5.5 in 6 *M* urea. The rate of hydrolysis of this substrate can be related to the amount of enzyme present[48] but values at zero time cannot be obtained by this method. (b) Optical Rotation: Since neither of the above procedures can be used for the monoacetyl-α-chymotrypsins, the denaturation was followed in a Rudolph Photoelectric Spectro-Polarimeter.[29] To 4 ml. of 12 *M* urea in the appropriate buffer (citrate or tris-maleate, 0.05 *M*) were added at zero time, 2 ml. of the enzyme (30 mg./ml.) and the optical rotation was recorded as a function of time at 23°.

Spectroscopic Studies.—The spectral changes at 245 mμ observed with the isolated intermediates AC-A and AC-I were followed on a Cary, Model 14, self-recording spectrophotometer. Approximately 4–5 mg. of the enzyme were dissolved in 1 ml. distilled water in a cuvette. The *p*H of these solutions was between 3 and 4 under which conditions the enzymes were isolated and stable. To the cuvette were added 2 ml. of the buffer (0.05 *M* tris-HCl and 0.05 *M* $CaCl_2$) to bring the *p*H to 8.0 and the absorbancy recorded at 245 mμ. The temperature was 20 ± 0.5°.

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