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Spectrophotometric Investigations of the Mechanism of α-Chymotrypsin-catalyzed Hydrolyses. Detection of the Acyl-enzyme Intermediate[1-3]

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Cinnamic acid substrates, which absorb strongly in the ultraviolet region, have been used to follow the kinetics of the acylation and deacylation steps of α-chymotrypsin-catalyzed hydrolyses. The reactions were carried out using stoichiometric concentrations of enzyme and substrate. The α-chymotrypsin-catalyzed hydrolyses of five labile cinnamic acid derivatives proceed *via* a fast acylation step followed by a slow deacylation step whose rate constant is identical for all five reactions, indicating the presence of a common intermediate. This intermediate, which is formed at the same rate as the departure of the leaving group from the cinnamic acid derivative, must be a covalent compound, *trans*-cinnamoyl-α-chymotrypsin. The difference spectrum (*vs.* enzyme) of this compound has been determined. From the position of its maximum absorption, this compound resembles the model compound O-cinnamoyl-N-acetylserinamide. The rate of the (alkaline) deacylation of *trans*-cinnamoyl-α-chymotrypsin in 7.74 *M* urea is practically identical with that of the model compound O-cinnamoyl-N-acetylserinamide. These data indicate that the acyl group of the acyl-enzyme is attached to an oxygen atom of the enzyme in the form of an ester linkage.

Introduction

The Mechanism of Enzyme Action.—This paper constitutes the first of several connected papers on the mechanism of action of the enzyme α-chymotrypsin. In these papers a number of approaches of physical organic chemistry has been applied to this enzymatic process. The enzyme α-chymotrypsin was chosen as a subject for mechanistic investigation for two reasons: (1) there is a wealth of information on the catalytic processes through which carboxylic acid derivatives may be hydrolyzed by non-enzymatic means and (2) the enzyme α-chymotrypsin is a crystalline organic compound whose reactions have been investigated in detail. Many enzymes contain a protein matrix and a non-protein coenzyme or prosthetic group, with which the enzymatic activity has been shown to be intimately connected. On the other hand, α-chymotrypsin and some other proteolytic enzymes are completely protein in nature; the mechanism of their catalytic processes must therefore involve protein components only. It is hoped in these investigations to elucidate the mechanism of action of an enzyme whose catalytic function resides solely within the protein molecule.

Chymotrypsin has a molecular weight of about 24,800, but has only one active site per molecule. This is shown by the fact that diisopropyl phosphorofluoridate reacts with chymotrypsin in a 1:1 stoichiometric reaction to give a crystalline product which contains one gram atom of phosphorus per mole and which is completely inactive enzymatically.[5] The object of the present investigations is to describe the active site and its action in as detailed a manner as possible. The active site is probably small compared to the entire enzyme, since even specific substrates are much smaller than the enzyme and since several hydrolytic enzymes have been cleaved of appreciable fractions of their total bulk with little or no loss of catalytic activity.[6] If the active site is indeed small, for example if it contains four or five groups which are necessary for catalytic action, it may lie within the province of the chemist to elucidate the nature of the active site of the enzyme and the mechanism of its catalysis.

Mechanism implies a complete description of all intermediates and all transition states in a chemical process. It is to this end that attention has been directed in the present papers. Since an enzyme is a catalyst, interest primarily revolves about the description of the particular set of catalytic functionalities of the active site and of their participation in transition states and intermediates of the enzymatic process. The specificity of the enzyme will be treated only indirectly. It is the contention of the present authors that the basic components of the active site are the catalytic entities; it may be said that the catalytic entities are the *necessary* condition for enzymatic action, although they are not a sufficient condition. On the basis that the catalytic components of the active site can be treated separately and that they are the components which are most amenable to investigation, the present experiments have been carried out.

In order to carry out such investigations of the catalytic components of the active site, reactions of α-chymotrypsin with so-called non-specific substrates have been investigated. This procedure lays the present experiments open to criticism by those biochemical purists who claim that reactions of proteolytic enzymes with substrates other than protein are irrelevant. But in fact it has been possible in many cases to show that the reactions of non-specific substrates parallel the esterase, amidase and proteolytic activity of proteolytic enzymes and in the present experiments care will be taken to indicate such parallelisms.

Many approaches have been used to elucidate the mechanism of enzyme action. Ultimately one might hope to reduce the problem of enzymatic action to the level where it could be solved by a

(1) This research was supported by grants from the National Institutes of Health.

(2) Paper X in the series, The Mechanism of Action of Proteolytic Enzymes; previous paper, M. L. Bender, E. T. Kaiser and B. Zerner, *J. Am. Chem. Soc.*, **83**, 4656 (1961).

(3) Some of this material was presented in preliminary form: G. R. Schonbaum, K. Nakamura and M. L. Bender, *ibid.*, **81**, 4746 (1959).

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(5) A. K. Balls and E. F. Jansen in "Advances in Enzymology," F. F. Nord (Editor), Vol. XIII, Interscience Publishers, Inc., New York, N. Y., 1952, p. 321.

(6) R. L. Hill and E. L. Smith, *Biochim. et Biophys. Acta*, **19**, 376 (1956); *J. Biol. Chem.*, **235**, 2332 (1960); G. E. Perlmann, *Nature*, **173**, 406 (1954).

quantum mechanical approach. The quantum mechanical approach has been advocated by Szent-Györgyi[7] recently for the interpretation of oxidation–reduction enzymes and has been applied to a number of enzymatic processes by the Pullmans.[8] Unfortunately, the present state of knowledge of most enzymatic processes is at such a low level that for many even a description of individual steps and intermediate compounds does not exist. Without such basic knowledge a quantum mechanical interpretation is not possible.

Another approach that has been used to investigate the active site of enzymes might be called topographical studies. Here the primary aim has been to produce a three-dimensional map of the enzyme surface and thus to identify its components and their action. To this end a vast number of substrates and inhibitors has been tested with the enzyme chymotrypsin, a formidable array of kinetic data has been accumulated for these systems,[9] and generalizations concerning the steric and structural specificity of this enzyme have been proposed.[10]

A number of investigations of chymotrypsin action have utilized experiments that have proved successful in the field of physical organic chemistry. Some of these approaches have been utilized in the present investigations: detailed kinetics of individual steps of the catalytic process, the effects of *p*H, deuterium oxide and structure on reactivity, and spectrophotometric identification of intermediates.

In the present paper the spectrophotometric detection of acyl-enzyme intermediates in α-chymotrypsin-catalyzed hydrolyses is described.

Chymotrypsin.—Chymotrypsin, as mentioned previously, is a proteolytic enzyme whose primary function is to hydrolyze peptides (amides), but it also cleaves esters as well as all other carboxylic acid derivatives.[9] It is a protein of molecular weight 24,800[11] containing one active site per molecule.[4] When the inactive phosphorylated enzyme mentioned earlier is degraded, it is found that the phosphorus atom resides on the hydroxyl group of the amino acid serine.[12] When chymotrypsin is treated with *p*-nitrophenyl acetate at low *p*H (at *p*H 4–5 compared with its *p*-optimum of 8) it is possible to isolate an acetyl-α-chymotrypsin.[13] This derivative is inactive as an enzyme, as is the diisopropylphosphoryl-enzyme, but in contrast to the diisopropylphosphoryl group, the acetyl group can be readily removed with nucleophiles at *p*H's around 8.[14] A number of analogs of acetyl-α-chymotrypsin have been prepared[15a]; noteworthy is trimethylacetyl-α-chymotrypsin which was successfully crystallized.[15b] Degradation of acetyl-α-chymotrypsin indicates that the acetyl group is bound to the hydroxyl group of the amino acid serine, paralleling the structural finding with the phosphoryl-enzyme.[16]

The hydrolysis of acetyl-L-tyrosine ethyl ester, a "specific" substrate for chymotrypsin, is inhibited by diisopropyl phosphorofluoridate and is also inhibited by *p*-nitrophenyl acetate.[17] This evidence indicates that the same part of the enzyme is involved in all these processes and that this active site involves the hydroxyl group of the amino acid serine. Treatment of chymotrypsin with radioactive diisopropyl phosphorofluoridate or Sarin and degradation of the labeled protein gives a series of phosphopeptides from which amino acid sequences have been obtained.[18] The amino acid sequence at the active site of chymotrypsin (and of a number of other enzymes) can be represented by the structure

$$\text{Gly}\begin{matrix}\text{Asp}\\ \text{Glu}\end{matrix}\text{Ser}\begin{matrix}\text{Gly}\\ \text{Ala}\end{matrix}$$

where Asp or Glu and Gly or Ala are alternatives in the sequence around the serine on which the radioactive phosphorus is found. It is evident from this sequence why chymotrypsin and a number of other enzymes have been classified as "serine proteinases"[19] and why so much attention has been paid to serine. However, since the integrity of the native structure of the enzyme must be maintained for enzymatic action, it is reasonable to believe that the sequence given above is only one component (experimentally the most accessible one) of the active site and that in general the active site must be composed of the juxtaposition of several (possibly helical) chains each of which contributes some functionality to the totality of what is called the active site. This defines the active site as a region among several chains which contribute nucleophiles, electrophiles and specificity loci.

In the hydrolysis of *p*-nitrophenyl acetate by chymotrypsin, an initial rapid liberation of one mole of *p*-nitrophenol per mole of chymotrypsin was observed, followed by a slow (zero order) reaction of the remaining substrate.[20] This behavior was interpreted in terms of a catalytic sequence involving two distinct steps in addition to the primary adsorption, a total of three steps.

(7) A. Szent-Györgyi, "An Introduction to Sub-Molecular Biology," Academic Press, Inc., New York, N. Y., 1960.

(8) For example, a general theory of enzymatic hydrolysis has been proposed: A. Pullman and B. Pullman, *Proc. Natl. Acad. Sci.*, **45**, 1572 (1959).

(9) See, for example, H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950); R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, **77**, 3365, 3370, 1886 (1955).

(10) H. T. Huang and C. Niemann, *ibid.*, **73**, 3223 (1951); G. E. Hein, R. B. McGriff and C. Niemann, *ibid.*, **82**, 1830 (1960); G. Hein and C. Niemann, *Proc. Natl. Acad. Sci., U. S.*, **47**, 1341 (1961); G. E. Hein and C. Niemann, *J. Am. Chem. Soc.*, in press (1962).

(11) W. J. Dreyer and H. Neurath, *J. Biol. Chem.*, **217**, 527 (1955); W. J. Dreyer, R. D. Wade and H. Neurath, *Arch. Biochem. Biophys.*, **59**, 145 (1955); P. E. Wilcox, E. Cohen and W. Tan, *J. Biol. Chem.*, **228**, 999 (1957); P. E. Wilcox, J. Kraut, R. D. Wade and H. Neurath, *Biochim. et Biophys. Acta*, **24**, 72 (1957).

(12) N. K. Schaffer, S. C. May, Jr., and W. H. Summerson, *J. Biol. Chem.*, **206**, 201 (1954).

(13) A. E. Balls and F. L. Alrich, *Proc. Natl. Acad. Sci., U. S.*, **41**, 190 (1955); A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245 (1956).

(14) A. K. Balls and C. E. McDonald, *ibid.*, **221**, 993 (1956).

(15) (a) C. E. McDonald and A. K. Balls, *ibid.*, **227**, 727 (1957); (b) A. K. Balls, C. E. McDonald and A. S. Brecher, Proceedings of the International Symposium on Enzyme Chemistry, Tokyo, Maruzen, 1958, p. 392.

(16) R. A. Oosterbaan and M. E. van Adrichem, *Biochim. et Biophys. Acta*, **27**, 423 (1958).

(17) T. Spencer and J. M. Sturtevant, *J. Am. Chem. Soc.*, **81**, 1874 (1959).

(18) N. K. Schaffer, S. C. May, Jr., and W. H. Summerson, *J. Biol. Chem.*, **202**, 67 (1953); H. K. Schaffer, L. Simet, S. Harshman, R. R. Engle and R. W. Drisko, *ibid.*, **225**, 197 (1957); F. Turba and G. Gundlach, *Biochem. Z.*, **327**, 186 (1955).

(19) B. S. Hartley, *Ann. Revs. Biochem.*, **29**, 45 (1960).

(20) B. S. Hartley and B. A. Kilby, *Biochem. J.*, **50**, 672 (1952); **56**, 288 (1954).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (1)$$
$$\qquad\qquad\qquad\qquad\quad + P_1$$

The initial rapid reaction can be followed by a stopped-flow method; using this method to follow the presteady-state portion of the reaction (the burst of *p*-nitrophenol), as well as the steady-state portion of the reaction, it was found that the kinetics are consistent with the scheme shown above.[21] The first step, which involves the rapid adsorption of the substrate on the enzyme, is assumed to have a rate constant k_1 greater than $10^6\ M^{-1}$ sec.$^{-1}$. The second step, which may be assumed to involve the acylation of the enzyme and the simultaneous liberation of *p*-nitrophenol (P_1), has a rate constant of 3.1 sec.$^{-1}$. The third step, deacylation, is postulated to involve the liberation of acetate (P_2) and reactivation of the enzyme, the rate constant being 0.0254 sec.$^{-1}$.[21]

A comparison of the rate constants of the base- and chymotrypsin-catalyzed hydrolyses of a series of substrates, an amide, an ethyl ester and a *p*-nitrophenyl ester, is instructive with regard to the stepwise process in eq. 1. Such a comparison is shown in Table I. In general it would be expected

TABLE I

A KINETIC COMPARISON OF BASE- AND α-CHYMOTRYPSIN-CATALYZED HYDROLYSES

Compound	Rel. k_2^{OH} [22] of acetates	$k_{chymotrypsin}$ of N-benzoyl-tyrosyl derivatives[23b] k_{cat}, sec.$^{-1}$	k_2, sec.$^{-1}$	k_3, sec.$^{-1}$
Amide	1	*0.24*	0.24	300
Ethyl ester	2,750	*200*	600	300
p-Nitrophenyl ester	315,000	*300*[a]	*>1000*	300

[a] N-Benzyloxycarbonyl-L-tyrosyl derivative. It would be expected that nitrophenyl and ethyl esters of the same acid would have identical turnover rate constants if k_3 were rate-controlling. This prediction is being pursued and will be the subject of a future publication. [b] Those rate constants which were experimentally determined are in italics. The others were calculated (see text).

that the series of compounds, amide, ester and *p*-nitrophenyl ester should exhibit reactivites in chymotrypsin catalysis parallel to those in basic catalysis since the acid portion of the series has been held constant and since there is ample evidence that more reactive members of a series of carboxylic acid derivatives are hydrolyzed more readily by chymotrypsin than less reactive members. It is seen that there is a fair degree of parallelism between base catalysis (k_2^{OH}) and chymotrypsin catalysis (k_{cat}) for the amide and ethyl ester pairs. However, this parallelism breaks down when comparing the *p*-nitrophenyl ester hydrolyses. The base-catalyzed hydrolysis of a *p*-nitrophenyl ester is over 100 times faster than that of an ethyl ester while the chymotrypsin-catalyzed hydrolysis of the *p*-nitrophenyl ester is only 50% faster than that of the ethyl ester. This discrepancy might be attributed to some inhibitory effect of the *p*-nitrophenyl substituent in enzymatic hydrolysis,[24] but more reasonably it might be attributed to the fact that the base-catalyzed hydrolysis does not involve an acyl-intermediate whereas the chymotrypsin-catalyzed hydrolysis does, as indicated in eq. 1. It may be shown that the (pH-independent) overall catalytic rate constant for the chymotrypsin-catalyzed reaction, k_{cat}, is related to the individual rate constants of acylation and deacylation, k_2 and k_3, by the expression[23]

$$1/k_{cat} = 1/k_2 + 1/k_3 \quad (2)$$

Knowing the k_{cat} for the three chymotrypsin-catalyzed reactions and k_3 for one of the reactions (k_3 is assumed to be the same for all three reactions since ES′ is identical in two reactions and is assumed to be very similar in the third) it is possible to calculate the acylation and deacylation rate constants, k_2 and k_3, shown in Table I. If one considers the steps controlled by the rate constants k_2 and k_3 to constitute a set of two consecutive first-order processes, k_2 is the rate-determining step of the over-all reaction for amide hydrolysis, k_3 is the rate-determining step for the *p*-nitrophenyl ester hydrolysis while both k_2 and k_3 contribute to the rate expression for the ethyl ester hydrolysis. This analysis implies that the acyl-enzyme intermediate (ES′) should be observable and isolable in the reaction of a *p*-nitrophenyl ester, as indeed has been shown (*vide supra*), that the acyl-enzyme intermediate might be observable but probably not isolable in the reaction of an ethyl ester, and that the acyl-enzyme intermediate should be observable or isolable in the reaction of an amide only with great difficulty. This analysis of course ignores consideration of the initial concentrations of enzyme and substrate and the equilibrium constant for the preliminary adsorption process. However, it does give a crude prediction for the experiments described in this and subsequent papers which concern themselves with the direct spectrophotometric detection of acyl-enzyme intermediates in chymotrypsin (and trypsin) reactions.

The evidence leading to the postulation of an acyl-enzyme intermediate (ES′) thus consists of three parts: (1) isolation of acyl-chymotrypsin compounds by reaction of nitrophenyl esters with chymotrypsin at low *p*H; (2) observation and kinetic analysis of an initial rapid liberation of one mole of *p*-nitrophenol per mole of chymotrypsin, followed by a slow reaction of the remaining *p*-nitrophenyl ester substrate; (3) a satisfactory kinetic correlation of the effect of structure on reactivity for amide, ethyl ester and *p*-nitrophenyl ester substrates. However, it must be admitted that the kinetic evidence is indirect and the isolation of the acyl-chymotrypsin intermediates was carried out at a *p*H somewhat removed from that usually used in the enzymatic process. Furthermore, even assuming that the phosphoryl- and acyl-chymotrypsins are true derivatives of the active site of the enzyme, the degradative conditions used to characterize the active site may lead to reactions which result in the production of artifacts.

(21) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).

(22) T. W. J. Taylor, *J. Chem. Soc.*, 2741 (1930); J. E. Potts, Jr., and E. S. Amis, *J. Am. Chem. Soc.*, **71**, 2112 (1949); and K. A. Connors, unpublished results, respectively.

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

(24) J. Roget and F. Calvet, *Rev. espan. fisiol.*, **16**, Suppl. 2, 215 (1960) (*C. A.*, **55**, 4601 (1961)).

The above considerations suggest the necessity of some experimental approach which will allow observation of the individual steps of the reaction and observation of the acyl-chymotrypsin intermediate without perturbation of the reaction system. Such an experimental approach is spectrophotometry. The use of spectrophotometry in the investigation of enzyme-substrate interactions has been developed to a high degree in studies of oxidation–reduction enzymes. The enzyme horse-radish peroxidase furnishes a classical example. The qualitative observations that the brown color of the enzyme solution changed to red upon the addition of hydrogen peroxide and further that the red color disappeared upon the addition of a reducing substrate provided qualitative evidence for the combination of an enzyme with its substrate to form an enzyme-substrate complex which was capable of reacting with a donor or reducing substrate.[25] These observations were followed by the discovery of a labile green compound preceding the formation of the red compound[26] and finally by measurements of the kinetics of these processes, after development of an apparatus capable of measurement of such fast reactions.[27] These investigations opened the era of studies of enzyme mechanisms by studying the kinetics of enzyme-substrate complex formation. In these studies, however, the course of the reaction was followed by measurement of changes in light absorption of the *enzyme* or *coenzyme* resulting from enzyme-substrate interaction. Since many enzymes (other than oxidation–reduction enzymes) do not possess any significant absorption connected with their active site, a different approach is needed in order to generalize this method. The approach used here is to exploit changes in the absorption of the *substrate* in order to detect enzyme-substrate intermediates.

Experimental

Materials.—N-*trans*-Cinnamoylimidazole was prepared by the reaction of *trans*-cinnamoyl chloride with imidazole in benzene solution; m.p. 133.5–134°.[28,29] *o*-Nitrophenyl, *m*-nitrophenyl, *p*-nitrophenyl and *p*-cresyl cinnamates were prepared from *trans*-cinnamoyl chloride (Eastman Kodak Co.) and the appropriate phenol in pyridine solution, followed by recrystallization from an aprotic solvent, in general, chloroform–hexane. Crude *p*-cresyl cinnamate was recrystallized twice from cyclohexane and once from petroleum ether (60–80°)–benzene (90:10). The recrystallization from cyclohexane appears to be very effective in removing the *m*-isomer, a major contaminant. The melting points of the purified products were: *o*-nitrophenyl cinnamate, 82–83°, lit.[30a] m.p. 84.5°; *m*-nitrophenyl cinnamate, m.p. 114–115° (Calcd. for $C_{15}H_{11}O_4N$: C, 66.91; H, 4.12; N, 5.20. Found: C, 67.04; H, 4.22; N, 5.24); *p*-nitrophenyl cinnamate, 146.5–147.5°, lit.[30a] m.p. 146°; *p*-cresyl cinnamate, 98.5–99.5°, lit.[30b] m.p. 100–101°.

O-Cinnamoyl-N-acetylserinamide was prepared from N-acetylserinamide[31] and cinnamoyl chloride in pyridine solution. After three recrystallizations from 50% methanol–water, the compound melted at 195–196°. Calcd. for $C_{14}H_{16}O_4N_2$: C, 60.86; H, 5.84; N, 10.14. Found: C, 60.65; H, 6.02; N, 10.36. O-Cinnamoyl-N-acetyltyrosinamide was prepared from chromatographically pure N-acetyl-L-tyrosine ethyl ester (Mann Research Laboratories) by first conversion to the amide,[32] m.p. 226.5–227.5°, and then treatment of the amide with cinnamoyl chloride in pyridine solution. Repeated recrystallization from 50% methanol-benzene gave colorless needles, m.p. 243–244.5°. Calcd. for $C_{20}H_{20}O_4N_2$: C, 68.17; H, 5.72; N, 7.95. Found: C, 68.61; H, 5.86; N, 7.90. *trans*-Cinnamic acid (Eastman Kodak Co. white label) was recrystallized two or three times from 20% ethanol–water; m.p. 134.5–135° with a slight softening at 133°. Cinnamaldehyde was purified by washing with saturated potassium carbonate solution to which a small amount of activated charcoal had been added, followed by drying and distillation under reduced pressure; $n^{25}D$ 1.6165 (lit.[33] $n^{20}D$ 1.6207). Urea (Baker analyzed reagent grade) was used directly or purified according to Steinhardt.[34] Kinetic results were consistent using either preparation.

The water used in enzymatic experiments was distilled water, redistilled in an all-glass apparatus from alkaline permanganate. Acetonitrile was an Eastman Kodak Co. spectro grade chemical and/or was purified by multiple distillation from phosphorus pentoxide followed by distillation from anhydrous potassium carbonate.[35] Buffer components including sodium acetate, citric acid, borax, potassium dihydrogen phosphate, disodium hydrogen phosphate and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) were of reagent grade quality. Sodium barbital was N.F. grade. Phosphate and borax buffers were prepared according to Kolthoff and Rosenblum.[36] Tris and carbonate buffers were prepared according to Bates and Bower.[37] Sodium chloride was of reagent grade quality.

A Radiometer *p*H meter, model 4b or 4c (precision ±0.002 *p*H unit), was used to determine the *p*H.

α-Chymotrypsin used in these experiments was either a twice- or thrice-crystallized preparation from Worthington Biochemicals Corp. Stock solutions (2×10^{-3} *M*) were prepared in water or in a suitable buffer. Enzyme solutions were ordinarily centrifuged to remove a small amount of lint which contaminates the Worthington product. For most experiments the normality of the enzyme solution was determined by the titration procedure with N-*trans*-cinnamoylimidazole as described previously.[28] In some early experiments the normality of the enzyme solution was based on the weight of the enzyme and a molecular weight for α-chymotrypsin of 24,800.[11] Experience indicates that the normality calculated on a weight basis is about 20% higher than that determined by the direct titration of the active sites of the enzyme solution.[28] In most cases the enzyme solution was titrated both before and after a given set of experiments in order to check possible deterioration of the enzyme during the course of a set of experiments. Where possible a given enzyme solution was used for a connected set of experiments and all experiments were conducted consecutively in order to obtain maximum internal consistency.

The preparation of *trans*-cinnamoyl-α-chymotrypsin was carried out with carefully standardized solutions of substrate and enzyme.[28] An excess of enzyme over substrate of about 5% was always allowed. The acetonitrile content of the final preparation could be as high as 15% provided the compound was used within 5 or 6 hours of its preparation.[38] A typical procedure is given: 2 ml. of a standardized

(25) D. Keilin and T. Mann, *Proc. Roy. Soc. (London)*, **B122**, 119 (1937).

(26) H. Theorell, *Enzymol.*, **10**, 250 (1941).

(27) B. Chance, *Science*, **92**, 455 (1940); B. Chance, *J. Biol. Chem.*, **151**, 553 (1943); B. Chance, *Arch. Biochem.*, **22**, 224 (1949)

(28) G. R. Schonbaum, B. Zerner and M. L. Bender, *J. Biol., Chem.*, **236**, 2930 (1961). The pure material has a high sheen when recrystallized from cyclohexane and melts to a completely colorless liquid.

(29) This same procedure has been reported by H. A. Staab, *Chem. Ber.*, **89**, 1927, 2088 (1956).

(30) (a) R. Anschütz, *Ber.*, **60**, 1322 (1927); (b) **18**, 1945 (1885).

(31) We acknowledge with thanks the gift of this compound from Dr. W. P. Jencks; *cf.* B. M. Anderson, E. H. Cordes and W. P. Jencks, *J. Biol. Chem.*, **236**, 455 (1961)

(32) D. W. Thomas, R. V. MacAllister and C. Niemann, *J. Am. Chem. Soc.*, **73**, 1548 (1951); these authors report m.p. 226–228° for the amide.

(33) R. P. Mariella and R. R. Raube, *J. Am. Chem. Soc.*, **74**, 521 (1952).

(34) J. Steinhardt, *J. Biol. Chem.*, **123**, 543 (1938).

(35) G. L. Lewis and C. P. Smyth, *J. Chem. Phys.*, **7**, 1085 (1939).

(36) I. M. Kolthoff and C. Rosenblum, "Acid–Base Indicators," the Macmillan Co., New York, N. Y., 1937, pp. 247–250.

(37) R. G. Bates and V. E. Bower, *Anal. Chem.*, **28**, 1322 (1956).

(38) Two factors must be kept in mind concerning the stability of *trans*-cinnamoyl-α-chymotrypsin: (1) the deacylation of the compound is *p*H dependent[33] and (2) the denaturation of the protein is a function of the percentage of organic solvent.

acetonitrile solution of N-*trans*-cinnamoylimidazole was transferred to a 10-ml. volumetric flask; a suitable quantity of a standardized stock enzyme solution (~500 μl.) was transferred to a 2-ml. volumetric flask. The acetonitrile solution of the substrate was diluted to the mark with *p*H 4–4.2 buffer (0.1 *M* total acetate) and 1 ml. of the resulting solution was rapidly transferred to the flask containing the enzyme. Under the conditions used, acylation of the enzyme is virtually instantaneous. To investigate reactions of the acyl-enzyme, 100 μl. of this solution was transferred to 3.00 ml. of the appropriate solution in the spectrophotometric cuvette. The acetonitrile concentration of the final reaction mixture is of course reduced to the order of 0.5% depending on the initial concentration of the reagent solution.

Spectra.—The spectra reported here were determined with either a Beckman DK-2 or a Cary recording spectrophotometer (models 11 and 14 PM), each of which was equipped with a thermostated cell compartment which maintained the temperature to ±0.1°; the Cary 14 PM spectrophotometer was equipped with 0–0.1 and 0–1.0 absorbance slide wires. All materials were carefully purified and all solutions standardized for spectral measurements. Scanning was usually carried out at the slowest speed for best accuracy. In general the conformance of a particular compound to a Beer's law relationship was assumed.

Difference spectra were determined both directly and by calculation. In the latter method absorbances were measured in the conventional way against a blank cuvette containing solvent only, and the resulting spectra were used to compare one solution with another, and thus to compute a difference spectrum. When we speak of the difference spectrum of state 2 *vs.* state 1, or of state 2 referred to state 1, we mean $\Delta A = A_2 - A_1$. When difference spectra were determined directly, care was taken to ensure that no artifacts were being observed because of high background absorptions resulting in spurious stray light effects.

The difference spectrum of *trans*-cinnamoyl-α-chymotrypsin *vs.* α-chymotrypsin was determined in the following manner: 3.00 ml. of a standardized solution of α-chymotrypsin[38] in *p*H 4.2 (0.1 *M* total acetate) buffer was equilibrated at 25.0° in the sample and reference cuvettes of the spectrophotometer (Cary 11, Beckman DK-2). A baseline was scanned and shown to be reproducible during the time of the experiment. Fifty μl. of purified acetonitrile was added to the reference cuvette and 50 μl. of a standard acetonitrile solution of N-*trans*-cinnamoylimidazole was added to the sample cuvette (at zero time). Under the conditions of the experiment, acylation is virtually instantaneous. The difference spectrum of *trans*-cinnamoyl-α-chymotrypsin *vs.* α-chymotrypsin was then scanned directly, since imidazole does not absorb in the region of interest. In these experiments, the final enzyme concentration was adjusted to be 5–10% greater than that of the substrate. The spectrum has been similarly determined several times at *p*H's between 4 and 5; the maximum enzyme "background" has been varied (depending on the machine used) between ~1 and 2.7 absorbance units, with consistent results.

Kinetics.—The kinetics of all reactions were followed spectrophotometrically in either the Beckman DK-2 or the Cary model 14 PM recording spectrophotometer. One of the following two experimental techniques was used in following the enzyme kinetics: (1) The buffer solution (3.00 ml.) was thermostated in the cell compartment for 15 to 30 min. A small volume (25–100 μl.) of an acetonitrile solution of the substrate was added and then the reaction was initiated by adding an aliquot of enzyme solution from the tip of a stirring rod and stirring vigorously for a few seconds. (2) The buffer solution (3.00 ml.) in a cuvette was thermostated in the cell compartment for 15–30 min. A small volume of enzyme solution was added and the reaction was initiated by adding a small volume of an acetonitrile solution of the substrate and stirring vigorously for a few seconds. The latter technique was used when non-enzymatic hydrolysis of the substrate was appreciable such as with N-*trans*-cinnamoylimidazole. The former technique was used with the nitrophenyl esters, in particular when large concentrations of substrate were utilized. Recording was started before initiation of the reaction when using the DK-2, and not later than 8 seconds after initiation of the reaction with the Cary.

The rates of liberation of the nitrophenols were recorded at the absorption maximum of either the nitrophenol or of the nitrophenoxide ion depending on the *p*H.[40] At these wave lengths, maximum absorbance changes are observed and no interference with enzyme absorption is encountered. Extinction coefficients of the nitrophenols under the conditions of these experiments were determined and agreed reasonably with those in the literature.[40] The acylation of α-chymotrypsin by N-*trans*-cinnamoylimidazole was followed by the rate of disappearance of the substrate at 335 mμ. The deacylation of *trans*-cinnamoyl-α-chymotrypsin was followed both by the rate of disappearance of the acyl-enzyme at 310 mμ and by the rate of appearance of cinnamate ion at 250 or 260 mμ. It has been shown that the rates obtained at these two wave lengths are closely concordant. In all cases but the last, the wave length selected is outside the range of enzyme absorption and thus no complications could arise from this source; 250 or 260 mμ was selected as the wave length for following the appearance of cinnamate ion as a compromise between the wave length of maximum absorption of cinnamate ion (269.5 mμ) and the wave length of minimum enzyme absorption (~249 mμ). Typical kinetic plots are shown in a subsequent paper.[39]

The (alkaline) rate of deacylation of *trans*-cinnamoyl-α-chymotrypsin was measured spectrophotometrically under pseudo-first-order conditions in hydroxide–chloride buffers, 7.74 *M* in urea, by following the decrease in absorbance (loss of acyl-enzyme) at 310 mμ. The appropriate 8 *M* urea buffer (3.00 ml.) was equilibrated in the spectrophotometer and the reaction was initiated by the addition of an aliquot (100 μl.) of the stock acyl-enzyme solution. Thus the reaction solution was 7.74 *M* in urea. The pseudo-first-order rate constants were obtained from "infinity" plots and/or by the method of Guggenheim.[41] The second-order (alkaline) rate constants were calculated using the measured *p*H and taking $pK_w = 14.00$.[42,43]

The alkaline hydrolyses of N-*trans*-cinnamoylimidazole, O-cinnamoyl-N-acetylserinamide and O-cinnamoyl-N-acetyltyrosinamide were followed spectrophotometrically, under pseudo-first-order conditions, at 335, 281 and 285 mμ, respectively. Borax buffers[37] were used for N-*trans*-cinnamoylimidazole; phosphate, carbonate and hydroxide-chloride buffers[37] were used for the amino acid derivatives.

Results and Discussion

Direct Spectrophotometric Detection of *trans*-Cinnamoyl-α-Chymotrypsin.—The first experiment that provided direct spectrophotometric detection of an acyl-enzyme intermediate was the α-chymotrypsin-catalyzed hydrolysis of *o*-nitrophenyl cinnamate. This substrate was chosen since it contains an acyl group and an alcohol group both of which absorb strongly in the ultraviolet or visible and whose absorbances do not completely overlap one another. When equimolar amounts of *o*-nitrophenyl cinnamate and α-chymotrypsin (about 10^{-4} *M*) reacted at *p*H's from 5.48 to 8.24, it was found (1) that the liberation of *o*-nitrophenol is practically complete in a few minutes (from observations at 350 mμ); and (2) the absorbance at 250 mμ decreases, reaching a minimum in that time which is required for formation of *o*-nitrophenol; the absorbance then slowly rises (over a period of time much greater than that for formation of *o*-nitrophenol) to a maximum value. The absorbance

(38) See L. Doub and J. M. Vandenbelt, *ibid.*, **69**, 2714 (1947) **71**, 2414 (1949) for a compilation of these spectra.

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

(40) See L. Doub and J. M. Vandenbelt, *ibid.*, **69**, 2714 (1947) **71**, 2414 (1949) for a compilation of these spectra.

(41) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd Edition, J. Wiley and Sons, Inc., New York, N. Y., 1961, p. 49.

(42) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Third Ed., Reinhold Publishing Corporation, New York, N. Y., 1958, p. 645.

(43) See ref. 39 for the experimental procedures utilized at high *p*H's.

at infinity is equal to the calculated sum of the *o*-nitrophenol and cinnamic acid/cinnamate ion absorbances. A typical example of this behavior is shown in Fig. 1. Since cinnamic acid is not appreciably formed by the time *o*-nitrophenol formation is complete, the decrease in absorbance at 250 mμ must correspond to the formation of a *trans*-cinnamoyl-α-chymotrypsin intermediate.

The behavior noted in the α-chymotrypsin-catalyzed hydrolysis of *o*-nitrophenyl cinnamate is also noted in the α-chymotrypsin-catalyzed hydrolyses of *m*-nitrophenyl and *p*-nitrophenyl cinnamates. In each case the liberation of the nitrophenol is completed very rapidly, at which time a minimum in the absorbance at 250 mμ is noted. The α-chymotrypsin-catalyzed hydrolysis of *N*-*trans*-cinnamoyl-imidazole is characterized by different spectrophotometric behavior. The disappearance of the substrate may be followed at 335 mμ; at equivalent concentrations of enzyme and substrate of about 10^{-4} *M* this disappearance is quite rapid, being complete is less than one minute. If one observes the reaction at about 250 mμ, an initial plateau is found, followed, after the time calculated for the disappearance of the substrate, by a rise in absorbance comparable to those noted in the nitrophenyl ester reactions. In the α-chymotrypsin-catalyzed hydrolysis of methyl cinnamate still another variant occurs.[44] An initial rise in absorbance is found followed by a slow fall. Thus it appears that the acylation and deacylation of α-chymotrypsin can be followed spectrophotometrically, with either maxima, minima or plateaus characterizing a portion of the reaction. The various types of behavior are dictated by the molar absorptivities (at a particular wave length) of all species present. From the present experiments it appears that the absorptions of conjugated systems such as those found in cinnamic acid derivatives are perturbed by the enzyme environment. This produces the happy result that the spectrum of the intermediate in this reaction is considerably different from that of either the substrate or the product, and presumably the same result may be predicted for other enzymatic processes involving highly absorbing substrates (see section on Spectra).

The results of the α-chymotrypsin-catalyzed hydrolysis of the nitrophenyl cinnamates can be interpreted in terms of eq. 1 where P_1 is the nitrophenol and P_2 is cinnamic acid. Thus these spectrophotometric experiments confirm in a direct manner the inferences obtained from kinetic and isolation experiments.

The question may arise as to whether ES, the Michaelis complex or ES′, the presumed acyl-enzyme intermediate, is being observed spectrophotometrically. The answer to this question seems to be straightforward since the minimum in Fig. 1 corresponds to that time when P_1, the nitrophenol, has been completely liberated. This means that the minimum must correspond to ES′, the acyl-enzyme intermediate, a covalent compound, and not to ES, the Michaelis complex.

It may be asked whether it is possible to observe ES by some spectrophotometric means. We have attempted to observe spectrophotometrically any behavior of the system before the formation of ES′ which might be interpreted in terms of another intermediate. No definite evidence has been found for any intermediate other than ES′. It would be expected of course that the formation of ES is very fast indeed[21] and that even if its absorbance were different from that of (E + S), the transformation from (E + S) to ES could not be observed because of the short time scale of the acylation reaction.[45]

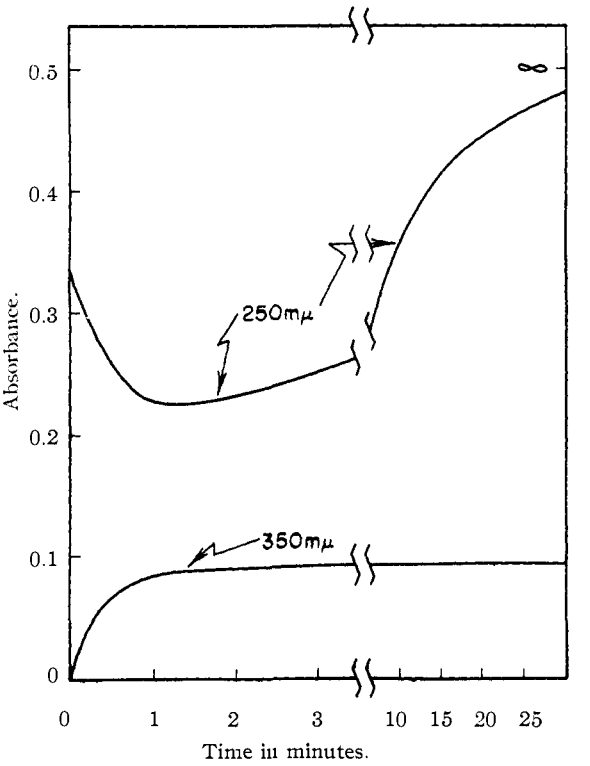

Fig. 1.—The α-chymotrypsin-catalyzed hydrolysis of *o*-nitrophenyl cinnamate at 25° in *p*H 6.2 phosphate buffers containing 10% acetonitrile; $[E]_0 = [S]_0 = 0.42 \times 10^{-4}$ *M*.

The spectrophotometric observation of an intermediate in the transformation of ES′ to P_2 would also be of interest. This reaction has also been examined and it was found that a precise isosbestic point occurs, indicating the absence of a further stable intermediate. Further comment on this point will be given in the section on Spectra.

Equimolar concentrations of enzyme and substrate were ordinarily used in the experiments described above so that the enzymatic process could be viewed in a stoichiometric manner. This procedure facilitated the use of the known molar absorptivities of the substrate and product to determine whether in fact stoichiometric reactions were being observed. Such a result was amply verified. If the reaction was carried out at a higher *p*H (~8) where deacylation occurs fairly rapidly, an additional aliquot of substrate could be added to the reaction solution at the conclusion of one reaction, and a second reaction could be followed. The second reaction would invariably reproduce the stoichiometry of the first reaction; however, since the product, cinnamic acid, is a competitive inhibi-

(44) M. L. Bender and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2550 (1962).

(45) H. Gutfreund, *Disc. Faraday Soc.*, **20**, 167 (1955).

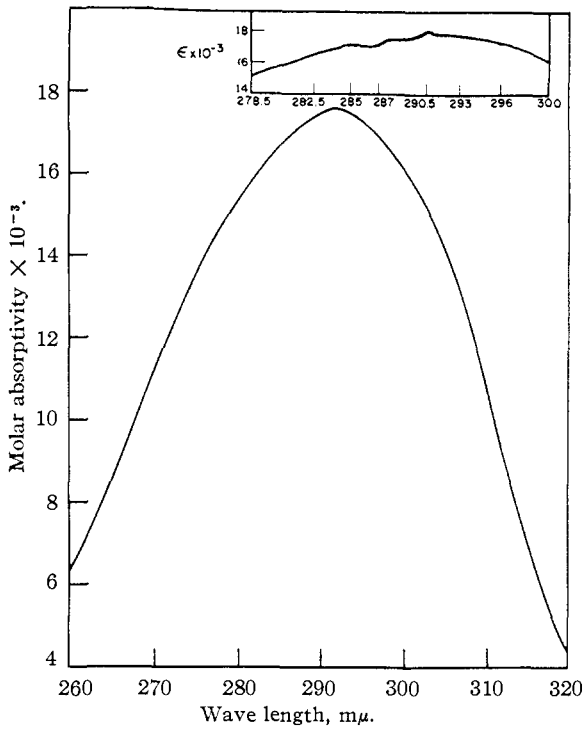

Fig. 2.—Difference spectrum of *trans*-cinnamoyl-α-chymotrypsin *vs.* α-chymotrypsin at *p*H 4.28. Inset shows the small "bumps" near the maximum on an expanded wave length scale.

tor in the reactions of any of the substrates mentioned above, the rate of the acylation process in a second reaction was slower than in the first. If the reaction was carried out at low *p*H where turnover (deacylation) is negligible; and if excess substrate (such as N-*trans*-cinnamoylimidazole) was present, four aliquots of enzyme could be added one after another, each producing the same stoichiometry, irrespective of the excess of substrate present. These experiments point to a stoichiometric reaction possessing an end-point which is independent of the concentration of the reacting species, implying that the end-point corresponds to 100% reaction and not to an equilibrium of less than 100% reaction.

Kinetics of Acylation.—From the appearance of nitrophenol or the disappearance of N-*trans*-cinnamoylimidazole, the kinetics of acylation of α-chymotrypsin by these substrates can be determined. These reactions were ordinarily carried out under second-order conditions, that is when concentrations of enzyme and substrates were of the same order of magnitude. The observed second-order rate constants correspond to k_2'/K_m ($= k_1k_2'/k_{-1}$). The theoretical justification for the observation of these second-order reactions is given in another paper of this series.[39] The results of these kinetic experiments are shown in Table II. It is seen that the observed second-order rate constants are extremely large, reflecting not only a large value for k_2', the acylation rate constant, but also a small value for K_m, the Michaelis constant. Table II indicates the excellent reproducibility of the kinetics as well as strict adherence to second-order kinetics at different ratios of [enzyme]/[substrate].

TABLE II

ACYLATION OF α-CHYMOTRYPSIN WITH SOME *trans*-CINNAMIC ACID DERIVATIVES AT 25.0°

$[E]_0 \times 10^6$, M	$[S]_0 \times 10^6$, M	$[E]_0/[S]_0$	$k_2'/K_m \times 10^{-3}$, M^{-1} sec.$^{-1}$	*p*H
	p-Nitrophenyl cinnamate[a,e]			
8.18	4.81	1.70	11.8	
20.2	4.76	4.25	11.0	
10.2	4.80	2.13	11.2	
		Av.	11.3 ± 0.4	8.34
	m-Nitrophenyl cinnamate[a,f]			
47.9	47.1	1.02	1.03	
77.8	45.9	1.69	1.20	
59.3	46.6	1.27	1.01	
		Av.	1.08 ± 0.08	8.28
	N-Cinnamoylimidazole			
18.6	10.65	1.75	4.54	
36.9	10.56	3.49	4.73	
18.6	10.65	1.75	4.55	
		Av.	4.60 ± 0.10[b,e]	7.32
36.9	10.56	3.49	2.41	
18.6	10.65	1.75	2.33	
40.4	7.85	5.14	2.37	
		Av.	2.37 ± 0.04[c,e]	5.23
36.9	10.56	3.49	2.99	
18.6	10.65	1.75	2.83	
		Av.	2.91 ± 0.08[d,e]	5.30

[a] Buffer, 0.05 *M* Tris containing 10% CH_3CN. [b] Buffer, 0.1 *M* phosphate containing 10% CH_3CN. [c] Buffer, 0.1 *M* acetate containing 10% CH_3CN. [d] Buffer, Tris–acetic acid: 0.048 *M* HOAc, 0.045 *M* Tris, 0.05 *M* $CaCl_2$ 10% CH_3CN. [e] 11.4% acetonitrile–water. [f] 10.2% acetonitrile–water.

Spectrum of *trans*-Cinnamoyl-α-chymotrypsin.—Both the qualitative and quantitative data presented above indicate that the acylation of α-chymotrypsin by labile derivatives of cinnamic acid occurs very readily, and furthermore that the deacylation of the acyl-enzyme intermediate appears to be a relatively slow process (*vide infra*). Therefore it should be possible to prepare the acyl-enzyme intermediate in a relatively stable form and determine its absorption (difference) spectrum. Although the deacylation of *trans*-cinnamoyl-α-chymotrypsin is a slow process relative to that of the acylation, it is nevertheless appreciable ($k_3 = 12.5 \times 10^{-3}$ sec.$^{-1}$) at *p*H's above 8.5. Since it was found that the deacylation reaction is *p*H dependent,[39] as are all chymotrypsin-catalyzed processes, it was decided to prepare the acyl-enzyme at *p*H ~4 and to determine its absorption spectrum at that *p*H.

The difference spectrum of *trans*-cinnamoyl-α-chymotrypsin *vs.* α-chymotrypsin at *p*H 4.28 is shown in Fig. 2. It is seen that the difference spectrum consists of an intense absorption due to the cinnamoyl group, with a maximum at about 292 mμ. However, the absorption curve is not symmetrical as are the curves of other cinnamic acid derivatives. Two small bumps can be discerned near the absorption maximum. It has been reported that a difference spectrum of acetyl-α-chymotrypsin *vs.* α-chymotrypsin exists, consisting of two small peaks, one at 290 mμ and the other in

the region of 280–285 mμ, presumably due to absorption differences in the enzyme on acylation.[46] This possibility may explain the small bumps in the difference spectrum of *trans*-cinnamoyl-α-chymotrypsin *vs.* α-chymotrypsin and is being investigated in this Laboratory at the present time.

Theoretically, the position of the wave length of maximum absorption of the cinnamoyl group on the enzyme might be equated to a particular kind of covalent linkage, for each kind of cinnamic acid derivative appears to possess a characteristic absorption maximum (Table III).

TABLE III

ABSORPTION SPECTRA OF *trans*-CINNAMOYL DERIVATIVES

Compound	Water λ_{max}, mμ	Water $\epsilon \times 10^{-4}$	10 *M* LiCl[g] λ_{max}, mμ	Isoöctane λ_{max}, mμ
Cinnamaldehyde[a]	292	2.44	..	278[d]
Cinnamic acid	278	2.15	..	..
Cinnamate ion	269.5	2.03	..	..
Methyl cinnamate[b]	279.5	2.21	..	..
o-Nitrophenyl cinnamate[c]	287.5	2.35	Insol.	281.5
m-Nitrophenyl cinnamate[c]	285	2.52	..	..
p-Nitrophenyl cinnamate[c]	293	2.63	..	..
O-Cinnamoyl-N-acetylserinamide[c]	281.5	2.43	286	~275[e]
O-Cinnamoyl-N-acetyl tyrosinamide[a]	285	2.79	290	Insol.
N-Cinnamoylimidazole[a]	307	2.52	~318[e]	295
Cinnamoyl-α-chymotrypsin[f]	292	1.77	..	..

[a] 1.6% CH_3CN. [b] 3% CH_3CN. [c] 10% CH_3CN. [d] R. P. Mariella and R. R. Raube, *J. Am. Chem. Soc.*, **74**, 521 (1952). [e] Very sparingly soluble. [f] Difference spectrum *vs.* α-chymotrypsin at *p*H 4.28. [g] Dr. E. M. Kosower has suggested that the polarity of a 10 *M* lithium chloride solution is as much greater than that of a water solution as the polarity of an isooctane solution is less than that of a water solution.

The acyl-enzyme has been described previously as either an *N*-acylimidazole derivative (of a histidine moiety of the enzyme) or as an ester derivative (of a serine moeity of the enzyme). However, the position of maximum absorption of the cinnamoyl group in *trans*-cinnamoyl-α-chymotrypsin corresponds to the absorption maximum of neither of the two model compounds N-*trans*-cinnamoylimidazole or O-cinnamoyl-N-acetylserinamide in aqueous solution. If the acyl-enzyme does indeed correspond to one of these possibilities it appears that the position of maximum absorption of the cinnamoyl group in the environment of the active site of α-chymotrypsin is perturbed from its position in aqueous solution. This is a reasonable conclusion since all ultraviolet spectra are subject to considerable solvent effects (Table III) and since it is known that the absorption maxima of a number of conjugated systems are shifted on the formation of non-covalent complexes with proteins or enzymes.[47,48] If one does assume that the acyl-enzyme is an ester derivative of a serine moiety of the enzyme, the position of the absorption maximum of the acyl-enzyme may be explained by saying that the environment of the cinnamoyl group on the enzyme surface is similar to that observed in 10 *M* lithium chloride as solvent. This result is in agreement with the fact that a carboxylate ion (of an aspartate residue) occupies a position adjacent to the serine moiety of the active site of the enzyme, creating an ionic atmosphere analogous to that present in a solution of 10 *M* lithium chloride. If one assumes that the acyl-enzyme is an *N*-acylimidazole derivative of a histidine moiety of the enzyme, the position of the absorption maximum of the acyl-enzyme may be rationalized only by saying that the environment of the cinnamoyl group resembles that in an isoöctane solution, a rather difficult extrapolation to make. Although no unambiguous assignment of structure of the acyl-enzyme can be made on the basis of the ultraviolet difference spectrum of *trans*-cinnamoyl-α-chymotrypsin, it is reasonable to say that the position of the absorption maximum is consistent with its assignment as an ester of a serine moiety of the enzyme.[49]

At *p*H 7 the position of maximum absorption of *trans*-cinnamoyl-α-chymotrypsin was again determined in order to ascertain whether spectral evidence could be obtained for the transfer of the cinnamoyl group to another group at higher *p*H. The reason for this experiment is that the *p*H data to be discussed later indicate the involvement of a group with a pK_a of approximately 7, presumably an imidazole group which may serve as a receptor of the cinnamoyl group at higher *p*H's. The spectral experiments carried out at *p*H 7 were difficult because of the rapid deacylation of the acyl-enzyme at that *p*H. The spectral evidence indicates that there is a small shift in the absorption maximum to 293 mμ, not much greater than experimental error. Therefore no conclusion, either positive or negative, concerning an O- to N-acyl transfer can be made.

One final comment is in order on the spectra of conjugated systems in the environment of proteins. Since, as discussed above, perturbation of the absorption spectra of conjugated systems occurs on the formation of non-covalent complexes with proteins or enzymes, one might expect a perturbation of the spectrum of a cinnamoyl group whether it is complexed with the enzyme, directly attached by a covalent linkage to the enzyme or indirectly attached (through a further saturated system) to the enzyme. This conclusion implies that a cinnamoyl group (or other conjugated system) introduced as a side chain into *any substrate* would undergo a spectral change when attached in any fashion to the

(46) J. F. Wootton and G. P. Hess, *Nature*, **188**, 726 (1960).

(47) DPNH forms complexes with liver alcohol dehydrogenase, liver lactic acid dehydrogenase and heart lactic acid dehydrogenase in which the absorption maximum has shifted to shorter wave lengths; J. van Eys, F. E. Stolzenbach, L. Sherwood and N. O. Kaplan, *Biochim. et Biophys. Acta*, **27**, 63 (1958.)

(48) A number of azo dyes form complexes with proteins in which the absorption maximum of the dye may be shifted to either shorter or longer wave length depending on the *p*H and the structure of the dye and of the protein; I. M. Klotz, R. K. Burkhard and J. M. Urquhart, *J. Am. Chem. Soc.*, **74**, 202 (1952).

(49) The absorption maximum of the model compound O-cinnamoyl-N-acetyltyrosinamide in 10 *M* lithium chloride is even closer to that of the acyl-enzyme than the absorption maximum of the serine model. From the spectral data, therefore, one cannot distinguish between a serine ester, and a threonine ester (which would be expected to have exactly the same spectral characteristics), and a tyrosyl ester.

enzyme, and thus make it feasible to follow individual steps of enzymatic processes.

Reactions of *trans*-Cinnamoyl-α-chymotrypsin.—The acyl-enzyme intermediate *trans*-cinnamoyl-α-chymotrypsin is easily prepared; furthermore its reactions may be followed readily by spectrophotometric means. Therefore detailed kinetic investigations of its reactions have been carried out. *trans*-Cinnamoyl-α-chymotrypsin should be formed in the α-chymotrypsin-catalyzed hydrolysis of any cinnamic acid derivative. Five such hydrolyses shown in Table IV have been carried out with N-*trans*-cinnamoylimidazole, *o*-nitrophenyl- *m*-nitrophenyl, *p*-nitrophenyl and *p*-cresyl cinnamates, all of which are substrates for α-chymotrypsin.

TABLE IV

RATES OF DEACYLATION OF α-CHYMOTRYPSIN-CATALYZED HYDROLYSES[a]

Substrate	$k_3 \times 10^2$, sec.$^{-1}$
N-*trans*-Cinnamoylimidazole	12.5 ± 0.5
o-Nitrophenyl cinnamate	12.5 ± .5
m-Nitrophenyl cinnamate	12.5 ± .5
p-Nitrophenyl cinnamate	12.5 ± .5
p-Cresyl cinnamate	12.5 ± .5

[a] $[E]_0 \simeq 1.05[S]_0$, $[S]_0 = 2\text{–}4 \times 10^{-5}$ *M*, 1.6–3% CH_3CN, *p*H 9.0.

The rates were followed spectrophotometrically at 310 mμ; in each case the very rapid (and sometimes almost instantaneous) acylation reaction is followed by a slower, first-order decay, the deacylation, whose rate constant is $12.5 \pm 0.5 \times 10^{-3}$ sec.$^{-1}$. The appearance of cinnamate ion at 260 mμ has the same rate as the disappearance of the acyl-enzyme intermediate at 310 mμ. Since the hydrolysis of *o*-nitrophenyl cinnamate has been shown to proceed through *trans*-cinnamoyl-α-chymotrypsin (whose deacylation is rate-controlling) the present data can best be interpreted in terms of the formation of the common intermediate, *trans*-cinnamoyl-α-chymotrypsin, in the hydrolysis of all five substrates.

The question may be raised as to whether the acyl-enzyme intermediate being investigated here is of the same type as that isolated by Balls. Therefore *trans*-cinnamoyl-α-chymotrypsin was prepared and isolated according to the method of Balls, *et al.*[50,51] The isolated acyl-enzyme gave a spectrum identical to that shown in Fig. 2 and its rate constant of deacylation was identical to that shown in Table IV.

Discussion of the effect of *p*H and of deuterium oxide on the deacylation of *trans*-cinnamoyl-α-chymotrypsin will be the subject of later papers in this series. However, the effect of a denaturing solvent, 7.74 *M* urea, will be discussed at this time. Although urea does not produce any profound change in the cinnamoyl absorption, it does produce a striking effect on the kinetics of deacylation.[52] The deacylation of *trans*-cinnamoyl-α-chymotrypsin is characterized[39] by dependence on a group with an apparent pK_a of 7.15 and independence of *p*H above 8.5, the region cited in Table IV. However, when *trans*-cinnamoyl-α-chymotrypsin is deacylated in 7.74 *M* urea, the reaction is transformed from an enzymatic hydrolysis to a non-enzymatic, base-catalyzed hydrolysis. The alkaline hydrolysis of denatured *trans*-cinnamoyl-α-chymotrypsin may be compared kinetically with the alkaline hydrolyses of a number of model compounds such as O-cinnamoyl - N - acetylserinamide, O - cinnamoyl-N-acetyltyrosinamide and N - *trans* - cinnamoylimidazole. The results of this comparison are shown in Table V.

TABLE V

ALKALINE HYDROLYSIS OF SOME *trans*-CINNAMOYL DERIVATIVES

	Cinnamoyl derivative of	7.74 *M* Urea: k_{OH}, *M*$^{-1}$ sec.$^{-1}$ [f]	7.74 *M* Urea: $k_{OH} \times 10^2$, *M*$^{-1}$ sec.$^{-1}$ [e,f]	7.74 *M* Urea: $\frac{k_{OH} \text{ of reacn. n}}{k_{OH} \text{ of reacn. 4}}$
1	N-Acetylserinamide[a]	3.65 ± 0.05	5.39 ± 0.35	1.3_1
2	N-Acetyltyrosinamide[a]	4.71 ± 0.1	6.45 ± 0.15	1.5_7
3	Imidazole[b]	1360 ± 10	1635 ± 35	399
4	α-Chymotrypsin[c]	.....	4.1 ± 0.3	
5	α-Chymotrypsin[d]	.....	4.0 ± 0.5	

[a] 1.6% CH_3CN, 25.6°, $pK_W = 13.97$. [b] 1.6% CH_3CN, 25.0°, $pK_W = 14.00$. [c] ~0.5% CH_3CN, 25.6°, $pK_W = 13.97$; acyl-enzyme prepared at *p*H 4.5. [d] ~0.5% CH_3CN, 25.0°, $pK_W = 14.00$; acyl-enzyme prepared at *p*H 7.22. [e] For the runs in 7.74 *M* urea, the ionic product of water was assumed to be the same as that in pure water. This is probably not valid, but the results should be approximately internally consistent. [f] The minimum number of runs involved in the determination of any rate constant was four, at 3 or 4 different *p*H's. The deviation conveys the total range of all measurements.

While *trans*-cinnamoyl-α-chymotrypsin in water hydrolyzes at a rate independent of *p*H in the region from *p*H 8.5 to 12.9,[39] the hydrolysis of this compound in 7.74 *M* urea is directly proportional to the hydroxide ion concentration and can be followed conveniently between *p*H 12 and 13. The hydrolyses of the cinnamoyl derivatives of serine, tyrosine and imidazole are also proportional to the hydroxide ion concentration. The rate constant of the alkaline hydrolysis of N-*trans*-cinnamoylimidazole in 7.74 *M* urea is considerably larger than that of the acyl-enzyme in 7.74 *M* urea (ratio = 399). On the other hand, the hydrolysis of O-cinnamoyl-N-acetylserinamide in 7.74 *M* urea has an alkaline rate constant which is very similar to that of the acyl-enzyme (ratio = 1.31). The latter rate ratio is quite similar to that found for the alkaline hydrolyses of the corresponding acetyl derivatives in 8 *M* urea (1.32).[31,54] Furthermore, the hydrolysis of O-cinnamoyl-N-acetyltyrosinamide has an alkaline rate constant in 7.74 *M* urea similar to that of the acyl-enzyme. Thus the acyl-enzyme in 7.74 *M* urea shows a reactivity considerably different from that of an acyl-imidazole and quite similar to that of either a seryl or a tyrosyl ester. The denaturing solvent 7.74 *M* urea has effected a transformation of *trans*-cinnamoyl-α-chymotrypsin from an enzymatic compound to a compound kinetically resembling an ordinary ester. The transformation by 7.74 *M* urea would be expected not to break

(50) A. K. Balls and F. L. Aldrich, *Proc. Natl. Acad. Sci.*, **41**, 190 (1955).

(51) A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245 (1956).

(52) Similar effects have been noted in a qualitative[53] and quantitative[39] manner in the deacylation of acetyl-chymotrypsin.

(53) G. H. Dixon, W. J. Dreyer and H. Neurath, *J. Am. Chem. Soc.*, **78**, 4810 (1956).

(54) The ratio of the alkaline rate constants of N-*trans*-cinnamoylimidazole and O-cinnamoyl-N-acetylserinamide is 373 (*cf.* ratio 389 for the corresponding acetyl derivatives[31]).

any covalent bonds and therefore one may conclude that the acyl-enzyme intermediate is also an ester.[55]

The *trans*-cinnamoyl-α-chymotrypsin first used in the above experiments was prepared at *p*H 4.5 prior to its denaturation. The possibility exists that if the acyl-enzyme were prepared at a higher *p*H prior to denaturation it might possess a cinnamoyl group attached to a different atom of the enzyme. Therefore *trans*-cinnamoyl-α-chymotrypsin was prepared at *p*H 7.22 and quickly transferred to an 8 *M* urea solution where the kinetics of its alkaline hydrolysis were determined. It was found that this procedure produced exactly the same alkaline rate constant as preparing the acyl-enzyme initially at *p*H 4.5 (Table V). This result again indicates that no appreciable quantity of an intermediate containing the cinnamoyl group attached to imidazole is observable in the deacylation step.

The hydrolytic behavior of *trans*-cinnamoyl-α-chymotrypsin in water and in 7.74 *M* urea defines in a succinct fashion the problem of enzymatic hydrolysis. In the former case one has an enzymatic process and in the latter case one has a corresponding non-enzymatic process. The hydroxide ion concentration must be approximately 10^6 greater in the latter case than in the former case in order to produce equivalent rates of reaction. Therefore one might say that the enzymatic deacylation is 10^6 more efficient than the non-enzymatic deacylation. What the enzyme does that hydroxide ion cannot do is to produce conditions in essentially neutral solution that will result in ester hydrolysis. A corollary of this argument is that the enzyme utilizes a water molecule to the exclusion of hydroxide ion in the deacylation reaction.

Conclusions

The α-chymotrypsin-catalyzed hydrolysis of labile cinnamic acid derivatives has proved to be of considerable utility in allowing the direct observation of the acylation and deacylation steps of the reaction, as well as the observation of the acyl-enzyme intermediate of the reaction. The question may be raised as to the pertinence of these observations to more normal reactions of α-chymotrypsin. On a weight basis it has been shown that one mole of α-chymotrypsin can be acylated by no more than one mole of N-*trans*-cinnamoylimidazole at low *p*H.[28] This result is in conformity with the observation that there is only one active site per α-chymotrypsin molecule, and implies that this special reaction takes place at the unique part of the enzyme, the active site. The cinnamic acid derivatives were designed with two structural requirements in mind: (1) possession of a conjugated system and (2) possession of a specificity of the chymotrypsin variety. The cinnamic acid derivatives are closely related structurally to β-phenylpropionic acid derivatives which possess the fundamental part of the specificity requirements for chymotrypsin.[9] Thus on specificity grounds one would expect that the stoichiometric reaction of cinnamic acid derivatives with α-chymotrypsin would take place at the active site. This conclusion is confirmed by the inhibition of the acylation with N-*trans*-cinnamoylimidazole by N-acetyl-L-tyrosine, a known competitive inhibitor for α-chymotrypsin.[56] The (second-order) acylation constant (k_2'/K_m) showed a significant decrease at *p*H 5.23 in the presence of N-acetyl-L-tyrosine, changing from $2.37 \pm 0.04 \times 10^3$ in the absence of inhibitor to 2.00×10^3 M^{-1} sec.$^{-1}$ in the presence of 5.32×10^{-5} *M* inhibitor. Thus one can say with considerable confidence that the reactions observed in this paper are not only α-chymotrypsin-catalyzed reactions, but also that they occur at *the* active site of the enzyme. Whether specific substrates follow the same pathway in their reactions will be the subject of further discussion in this series.[57]

Only one intermediate, the acyl-enzyme in which the acyl (*trans*-cinnamoyl) group is attached to an oxygen atom of the enzyme, is spectrophotometrically observable in the reactions investigated here. The pathway of these catalyses is therefore that described by eq. 1 in agreement with previous kinetic and isolation experiments. Neither the enzyme-substrate complex, ES, nor any variant of the acyl-enzyme is spectrophotometrically observable.

Spectrophotometric changes on changing the *p*H of the solution from 4 to 9 were interpreted by earlier workers to indicate the transformation of the acyl-enzyme intermediate, monoacetyl-α-chymotrypsin, into another form of the acyl-enzyme.[58] Kinetic evidence substantiated this claim; however, it was pointed out that the kinetic (and spectrophotometric) evidence for a second form of the acyl-enzyme stems not from the transformation of the normal intermediate in chymotrypsin-catalyzed reactions, acetyl-chymotrypsin-I, but rather from the transformation of another intermediate, acetyl-chymotrypsin-A.[59] Subsequent studies indicated that the spectrophotometric observations were based on an artifact which occurred with α-chymotrypsin alone,[60] and finally this artifact was shown to be due to light scattering due to precipitation and re-solution of the protein during the *p*H transformation described above.[61] Observations made in these laboratories are in full agreement with the explanation of an artifact occurring during such treatment.[62] When monoacetyl-α-chymotrypsin prepared according to the procedure of Balls,[13,61] in *p*H 4 buffer was changed to *p*H 8 by the addition of a Tris buffer containing calcium chloride, a visible precipitate was immediately

(55) This conclusion is in agreement with experiments carried out on the acylation of α-chymotrypsin with *p*-nitrophenyl acetate by H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci., U. S.*, **42**, 719 (1956). In this reaction it was found that protons are absorbed from the solvent, a result which is not consistent with any appreciable (observable) nitrogen acylation.

(56) D. W. Thomas, R. V. MacAllister and C. Niemann, *J. Am. Chem. Soc.*, **73**, 1548 (1951); R. J. Foster and C. Niemann, *Proc. Natl. Acad. Sci.*, **39**, 999 (1953).

(57) For a detailed discussion of the relationship of the cinnamoyl substrates to specific substrates, see M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2582 (1962).

(58) G. H. Dixon and H. Neurath, *ibid.*, **79**, 4558 (1957).

(59) M. A. Marini and G. P. Hess, *ibid.*, **81**, 2594 (1959); *Nature*, **184**, 113 (1959).

(60) T. Spencer and J. M. Sturtevant, *J. Am. Chem. Soc.*, **81**, 1874 (1959).

(61) J. F. Wootton and G. P. Hess, *ibid.*, **82**, 3789 (1960).

(62) We acknowledge with thanks the gift of this material from Dr. G. P. Hess.

formed (resulting in an apparent increase in absorbance at all wave lengths down to 245 mμ) which then slowly redissolved (resulting in an apparent decrease in absorbance with a half-life of approximately five minutes). However, at no time did any preparation of *trans*-cinnamoyl-α-chymotrypsin undergo these precipitation and redissolution phenomena.[63] Thus there appears to be only one experimentally verifiable type of acyl-chymotrypsin intermediate, which is the subject of the present paper.

Both the spectral data, which are somewhat ambiguous, and the kinetic data in 7.74 *M* urea, which are relatively unambiguous, indicate that the acyl group of the acyl-enzyme is attached to an oxygen atom of the enzyme in the form of an ester linkage. This evidence supports completely the suggestions made from isolation experiments that the acyl group is attached to the oxygen atom of a serine moiety of the enzyme.[16] The occurrence of this acyl-enzyme intermediate in the α-chymotrypsin-catalyzed hydrolyses of many labile esters has now been demonstrated without doubt. The occurrence of a similar intermediate in the α-chymotrypsin-catalyzed hydrolysis of a non-labile ester and in a trypsin-catalyzed hydrolysis will be reported in subsequent papers in this series.

(63) It has been reported that acetyl-chymotrypsin loses its acetate residue (as measured by ^{14}C activity) at a much slower rate than the rate of appearance of enzymic activity. This observation by T. Viswanatha and W. B. Lawson, *Arch. Biochem. Biophys.*, **93**, 128 (1961), implies that some acylation at other than the active site occurs, an observation confirmed in this Laboratory. However, this complication apparently does not apply to the more specific cinnamoyl systems.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NORTHWESTERN UNIVERSITY, EVANSTON, ILL.]

The Formation of an Acyl-enzyme Intermediate in the α-Chymotrypsin-catalyzed Hydrolyses of Non-labile *trans*-Cinnamic Acid Esters[1-3]

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The α-chymotrypsin-catalyzed hydrolyses of methyl and benzyl *trans*-cinnamates have been investigated under pseudo-first-order conditions in which the enzyme concentration is much greater than the substrate concentration. Under these conditions, the acylation and deacylation reactions can be described as two consecutive first-order reactions. In each enzymatic process, unequivocal evidence has been obtained for the involvement of *trans*-cinnamoyl-α-chymotrypsin as an intermediate in the reaction, both from rate measurements and absorption spectral characteristics. A quantitative fit of the absorbance *vs.* time curve for the α-chymotrypsin-catalyzed hydrolysis of methyl cinnamate was obtained using the measured molar absorptivities of reactant, intermediate and product, together with the independently determined (apparent) rate constant of acylation and the rate constant of deacylation. In addition, zero-order kinetics of the methyl cinnamate system enabled the calculation of the Michaelis constant for acylation and the (true) acylation rate constant. The ratio of these constants is consistent with that obtained directly from the first-order kinetics. It is concluded that the formation of an acyl-enzyme intermediate is not an artifact of α-chymotrypsin catalyses resulting from the lability of the ester function, but is part of the general mechanism of such catalyses.

Introduction

The hydrolysis of labile acyl derivatives (*e.g.*, nitrophenyl esters) is catalyzed by α-chymotrypsin in a three-step process: (i) adsorption of the substrate on the enzyme; (ii) acylation of the enzyme with the release of the phenol; and (iii) deacylation of the acyl-enzyme giving the carboxylic acid product and regenerating the enzyme.[5] In other papers of this series[6,7] the evidence supporting the involvement of the acyl-enzyme intermediate, *trans*-cinnamoyl-α-chymotrypsin, in the enzyme-catalyzed hydrolyses of five labile *trans*-cinnamic acid derivatives (*o*-, *m*-, *p*-nitrophenyl cinnamates, *p*-cresyl cinnamate and N-cinnamoylimidazole) has been thoroughly documented.

However, it has been maintained on the basis of kinetic arguments that the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate[8] does not proceed through a hippuryl-enzyme intermediate and (by implication) that the α-chymotrypsin-catalyzed hydrolyses of other non-labile substrates also do not involve acyl-enzyme intermediates.[9] With a view to establishing the generality of acyl-enzyme formation in the α-chymotrypsin-catalyzed hydrolysis of carboxylic acid derivatives, we therefore investigated the enzyme-catalyzed hydrolysis of two non-labile (alkyl) esters of *trans*-cinnamic acid, methyl and benzyl cinnamate. An account of this work is given in the present paper.

Experimental

Materials.—α-Chymotrypsin (3 × crystd., salt-free) was obtained from Worthington Biochemical Corporation and was used without further purification. Stock solutions ($1.5 - 3 \times 10^{-3}$ *M*) of the enzyme were prepared in appropriate buffers. All enzyme solutions were centrifuged to remove traces of lint and (at the higher concentrations) insoluble protein. The normality of the enzyme solutions was determined by titration[10] at the beginning and end of

(1) This research was supported by grants from the National Institutes of Health.

(2) Paper XI in the series, The Mechanism of Action of Proteolytic Enzymes; previous paper, M. L. Bender, G. R. Schonbaum and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2540 (1962).

(3) Some of the results of this paper have been presented previously: M. L. Bender and B. Zerner, *ibid.*, **83**, 2391 (1961).

(4) Alfred P. Sloan Foundation Research Fellow.

(5) For a detailed list of references, see ref. 6, 7.

(6) M. L. Bender, G. R. Schonbaum and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2540 (1962).

(7) M. L. Bender, G. R. Schonbaum and B. Zerner, *ibid.*, **84**, 2562 (1962).

(8) S. A. Bernhard, W. C. Coles and J. F. Nowell, *ibid.*, **82**, 3043 (1960).

(9) M. L. Bender and W. A. Glasson, *ibid.*, **82**, 3336 (1960), also obtained kinetic results in the enzyme-catalyzed methanolysis of N-acetyl-L-phenylalanine methyl ester which do not appear to be readily reconciled with the formation of an acyl-enzyme intermediate in the reaction.

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