philized. A total of 1.3 g. of crude protected nonapeptide was reduced in this manner and the product, assayed by the Coon method,[15] possessed an activity of approximately 10,000 avian depressor units.

Purification and Isolation of the Active Product.—The lyophilized crude product was placed in the first 10 tubes of the all-glass automatic countercurrent distribution apparatus[27] and distributed in the system *sec*-butyl alcohol–0.1% acetic acid. The progress of the purification was followed by determining the Folin color[28] and the biological activity of selected tubes. After 900 transfers the activity was concentrated in a single peak (K = 0.48) and was almost completely separated from a slower moving inactive component. The contents of the tubes with the active material were combined, concentrated in a rotary evaporator at a temperature below 30° and lyophilized to give 135 mg. of a white fluffy solid. The biological assays were performed on this solid and the results are given in Table I. The synthetic material possessed the specific rotation $[\alpha]^{22}D$ −33° (c 0.57, 0.1 N acetic acid).

Two mg. of the synthetic product and a similar amount of natural oxytocin were applied separately in pyridine–acetic acid buffer at pH 5.6 to a strip of Whatman No. 1 filter paper for electrophoresis, and 400 v. was applied for 24 hours at 5°. The material was stained on the paper by the method of Durrum[29] in which the dye, brom phenol blue, in ethyl alcohol saturated with mercuric chloride is employed. Both compounds, oxypressin and oxytocin, gave a single spot and exhibited the same mobility.

(27) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

(28) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(29) E. L. Durrum, THIS JOURNAL, **72**, 2943 (1950).

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Oxygen-18 Studies of the Mechanism of the α-Chymotrypsin-catalyzed Hydrolysis of Esters[1]

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An investigation of the mechanism of the α-chymotrypsin-catalyzed hydrolysis of esters has been made utilizing oxygen-18 techniques. The carbonyl oxygen exchange accompanying hydrolysis, the mode of fission and the alcoholysis accompanying hydrolysis were studied. The α-chymotrypsin-catalyzed hydrolysis of esters was found to differ from non-enzymatic (alkaline) hydrolysis with respect to carbonyl oxygen exchange during the hydrolytic process. Such oxygen exchange was found to occur during the alkaline hydrolysis of methyl β-phenylpropionate-*carbonyl*-O^{18} and benzoyl-L-phenylalanine ethyl ester-*carbonyl*-O^{18}, but not during the α-chymotrypsin-catalyzed hydrolysis of these esters. However, both the α-chymotrypsin-catalyzed and base-catalyzed hydrolytic reactions occur by means of acyl–oxygen fission. This was demonstrated in the enzymatic case by means of the hydrolysis of methyl β-phenylpropionate-*alkoxyl*-O^{18}. Both methyl β-phenylpropionate-*alkoxyl*-O^{18} and benzoyl-L-phenylalanine ethyl ester underwent simultaneous hydrolysis and methanolysis in the presence of α-chymotrypsin, the rate of methanolysis being greater than that of hydrolysis. The alcoholysis may be considered another example of an enzymatic exchange reaction. The above results are consistent with a mechanism of α-chymotrypsin action involving an acyl–enzyme intermediate.

Introduction

The report is the third in a series of investigations designed to explore the mechanism of action of a typical endopeptidase, α-chymotrypsin.[3,4] These studies are concerned not with the elucidation of multi-enzymatic systems, but rather with the determination of the detailed mechanism of individual acts by a hydrolytic enzyme, including a description of all intermediates in the process with the twin aims of elucidating the structure of the "active site" on the enzyme and of attempting the synthesis of a compound capable of producing enzymatic hydrolysis. The general approach of these investigations is to compare enzymatic and non-enzymatic hydrolyses from a mechanistic viewpoint. Following this procedure, it is hoped that the methods and results of studies of the mechanism of non-enzymatic hydrolysis, which are relatively complete,[5] can be applied to their enzymatic counterparts. The assumption is made here, of course, that enzymatic catalysis is explicable in chemical terms and that non-enzymatic hydrolysis is the proper point of departure for such an explanation. The methods that have yielded the most information in elucidating the mechanism of non-enzymatic hydrolysis have been kinetic studies, the effect of structure on reactivity and isotopic studies involving oxygen-18 for cleavage and exchange experiments. This report concerns an investigation of the mechanism of the α-chymotrypsin-catalyzed hydrolysis of esters utilizing oxygen-18 techniques. The carbonyl oxygen exchange accompanying hydrolysis, the mode of fission and the alcoholysis accompanying hydrolysis have been studied.

(1) This investigation was aided by research grant G-3787 from the National Institutes of Health, U. S. Public Health Service.

(2) From the Ph.D. thesis of K. C. K.

(3) Previous papers in this series: (a) M. L. Bender, R. D. Ginger and K. C. Kemp, THIS JOURNAL, **76**, 3350 (1954); (b) M. L. Bender and B. W. Turnquest, *ibid.*, **77**, 4271 (1955).

(4) A summary of previous studies on α-chymotrypsin is given by R. M. Herriott in "Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, Md., 1954, pp. 24–37.

(5) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell Univ. Press, Ithaca, N. Y., 1953, Chap. XIV; J. Hine, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1956, Chaps. 12 and 13.

Experimental

Materials.—α-Chymotrypsin was an Armour and Co., salt-free preparation. The concentration of α-chymotrypsin was determined by measuring the optical density at 282 mμ in a Beckman DU spectrophotometer. A standard calibration curve was obtained by determining the enzyme concentration by a micro-Kjeldahl procedure.[6] Methanol (Baker and Adamson Co.) was distilled, b.p. 64°. The oxygen-18 labeled esters listed in Table I were prepared by conventional means.

Kinetics of Carbonyl Oxygen Exchange during Alkaline Hydrolysis.—The rates of the alkaline hydrolysis of methyl and ethyl β-phenylpropionate-*carbonyl*-O^{18} and benzoyl-L-

(6) A. J. Hiller, J. Plazen and D. D. Van Slyke, *J. Biol. Chem.*, **176**, 1401 (1948).

TABLE I

PREPARATION OF OXYGEN-18 LABELED ESTERS

Ester	B.p.[d] °C.	Mm.	$n^{20}D$	$n^{20}D$ (lit.)
	Carbonyl-O^{18} labeled			
Methyl β-phenylpropionate[a]	91–92	4	1.5022	1.5030[8]
Ethyl β-phenylpropionate[a]	82	0.7	1.4947	1.4947[3b]
Benzoyl-L-phenylalanine methyl ester[c]	81–83[e]			81[9e]
Benzoyl-L-phenylalanine ethyl ester[c]	103.2–103.7[e]			101.5–102[10e]
	Alkoxyl-O^{18} labeled			
Methyl β-phenylpropionate[b]	57–59	0.1	1.5029	

[a] Prepared from β-phenylpropionic acid-*carboxyl*-O^{18}[7] through the acid chloride. [b] Prepared from the acid chloride and methanol-O^{18} which was kindly supplied by Dr. K. B. Wiberg, University of Washington. [c] Prepared from benzoyl-L-phenylalanine-*carboxyl*-O^{18}[7] by direct esterification. [d] Distillation was effected with a 25-cm. column packed with glass helices. [e] Melting point.

phenylalanine ethyl ester-*carbonyl*-O^{18} were studied in aqueous methanol at 25.04 ± 0.02°. In each case, the initial concentrations of base and ester were equal. At appropriate time intervals, 5.0-ml. samples were removed and pipetted into excess standard hydrochloric acid. The excess acid was then titrated to the phenolphthalein end-point with standard sodium hydroxide.

The kinetics of the carbonyl oxygen exchange of these three esters during alkaline hydrolysis were studied under conditions identical to those described above except that they were performed on a larger scale. At predetermined times, corresponding to 25, 50, 70 and 80% hydrolysis, aliquots of the reaction mixture were removed and quenched in excess hydrochloric acid. Excess sodium bicarbonate was added and the solution was extracted with methylene chloride. The recovered methyl and ethyl β-phenylpropionates were purified by distillation in a micro-distillation apparatus.[11] The recovered benzoyl-L-phenylalanine ethyl ester was purified by recrystallization from aqueous ethanol; the melting points of the recovered benzoyl-L-phenylalanine ethyl ester differed from the starting material and indicated that methanolysis occurred during the hydrolysis. Melting points of the recovered ester are given in the section on alcoholysis. After the purification, the esters were analyzed for their oxygen-18 content.

Carbonyl Oxygen Exchange during Enzymatic Hydrolysis.—The carbonyl oxygen exchange during the α-chymotrypsin-catalyzed hydrolysis of methyl β-phenylpropionate-*carbonyl*-O^{18} and benzoyl-L-phenylalanine methyl and ethyl esters *carbonyl*-O^{18} was studied by analyzing the unhydrolyzed ester for oxygen-18 after 50% and/or 80% hydrolysis. The times required for these extents of hydrolysis were determined by titration to a constant *p*H.[12] A Beckman model G *p*H meter equipped with external electrodes was used. The experiments used for the exchange studies were similar to the kinetic experiments except that larger volumes of solution were used and the concentration of the buffer was increased to keep the *p*H constant during the hydrolysis. A typical experiment was performed as follows: methyl β-phenylpropionate-*carbonyl*-O^{18} (100 mg.) dissolved in 10.0 ml. of methanol, 30.0 ml. of 0.05 *M* phosphate buffer (*p*H 7.8) and 100 mg. of α-chymotrypsin in 10.0 of redistilled water were placed in a 100-ml. volumetric flask and diluted to the mark with redistilled water. The reaction was allowed to proceed for 65 min., corresponding to 50% reaction. The reaction was stopped by precipitating the enzyme with 10 ml. of trichloroacetic acid solution (1.4 g. acid/ml.). Excess sodium bicarbonate was added and the solution was filtered. The unhydrolyzed ester was recovered by extraction with methylene chloride, distilled (b.p. 91° (3 mm.)) and analyzed for its oxygen-18 content.

Alcoholysis.—The hydrolysis of benzoyl-L-phenylalanine ethyl ester by base (0.004595 *M*) in 50% aqueous methanol and the α-chymotrypsin-catalyzed hydrolysis of this ester in 30% aqueous methanol were accompanied by methanolysis. At various extents of hydrolysis, the unhydrolyzed ester was recovered. Identification of the recovered material was carried out by means of mixed melting points and infrared spectra. A Perkin-Elmer model 21 double beam recording infrared spectrophotometer was used with a single sodium chloride cell of 0.1-mm. thickness.

The methanolysis accompanying the α-chymotrypsin-catalyzed hydrolysis of methyl β-phenylpropionate was studied using oxygen-18. Methyl β-phenylpropionate-*alkoxyl*-O^{18} was hydrolyzed to 50% completion in 15% methanol. The *p*H of the solution was 7.8 and was 0.033 *M* in phosphate buffer; the concentration of α-chymotrypsin was 9 mg./ml. The reaction was quenched and the unhydrolyzed ester was recovered by extraction with methylene chloride and analyzed for oxygen-18.

Determination of Enzymatic Mode of Fission.—The α-chymotrypsin-catalyzed hydrolysis of methyl β-phenylpropionate-*alkoxyl*-O^{18} was carried out under the reaction conditions given in the previous section. After the unhydrolyzed ester had been extracted with methylene chloride, the aqueous solution was acidified and the β-phenylpropionic acid was extracted with methylene chloride. The recovered acid was distilled in the micro-distillation apparatus (m.p. 44–46°) and analyzed for oxygen-18.

Oxygen-18 Analysis.—Analysis of oxygen-18 in the organic compounds used in this investigation was accomplished by converting the oxygen atoms of the labeled compounds to carbon dioxide and analyzing the carbon dioxide in a Consolidated-Nier model 21-201 isotope ratio mass spectrometer.[13] The following equations were used to calculate the atom fraction O^{18} where

n = mm. blank.
y = mm. pyrolysis sample − blank.
R_u = mass spectrometer reading for the 46/44 ratio of unknown CO_2.
R_s = mass spectrometer reading for the 46/44 ratio of standard CO_2.

Atom fraction O^{18} in the carbonyl (or alkoxyl) position of an ester in which only two oxygen atoms are present, one of which is labeled, is

$$\frac{1.998(R_u/R_s) - 1.4978 + 0.9980(n/y)((R_u/R_s) - 1)}{(R_u/R_s) + 244.10} \quad (1)$$

Atom fraction O^{18} in the carbonyl position of an ester in which three atoms of oxygen per molecule are present, only the carbonyl being labeled, is

$$\frac{2.996(R_u/R_s) - 2.4959 + 0.9980(n/y)((R_u/R_s) - 1)}{(R_u/R_s) + 244.10} \quad (2)$$

Results

Carbonyl Oxygen Exchange during Alkaline and α-Chymotrypsin-catalyzed Ester Hydrolysis.—The kinetic data for the alkaline hydrolysis of methyl and ethyl β-phenylpropionate and benzoyl-L-

(7) M. L. Bender and K. C. Kemp, THIS JOURNAL, **79**, 116 (1957).
(8) A. I. Vogel, *J. Chem. Soc.*, 654 (1948).
(9) J. E. Snoke and H. Neurath, *Arch. Biochem.*, **21**, 351 (1949).
(10) S. Kaufman and H. Neurath, *ibid.*, **21**, 437 (1949).
(11) M. J. Babcock, *Anal. Chem.*, **21**, 632 (1949).
(12) G. W. Schwert, H. Neurath, S. Kaufman and J. Snoke, *J. Biol. Chem.*, **172**, 221 (1948).
(13) See reference 7 for a discussion of the precision of the method and of the isotopic dilution due to a blank reaction. The derivation of eq. 1 and 2 may be found in the Ph.D. thesis of K.C.K., Illinois Institute of Technology, 1956.

phenylalanine ethyl ester in aqueous methanol are presented in Fig. 1 and the second-order rate constants are shown in Table II. Examination of the plots of the hydrolysis of the two ethyl esters reveals a drift in the rate constant which is not evident in the plot of the hydrolysis of the methyl ester. As will be shown later, this drift in the case of the ethyl esters is due to alcoholysis, converting the ethyl esters to methyl esters. In order to determine the rate constants for the hydrolysis of the ethyl esters, it is essential, therefore, to extrapolate to zero time. However, for purposes of calculation of the oxygen exchange, it is desirable to determine an average rate constant; such average hydrolytic rate constants are tabulated for the ethyl esters.

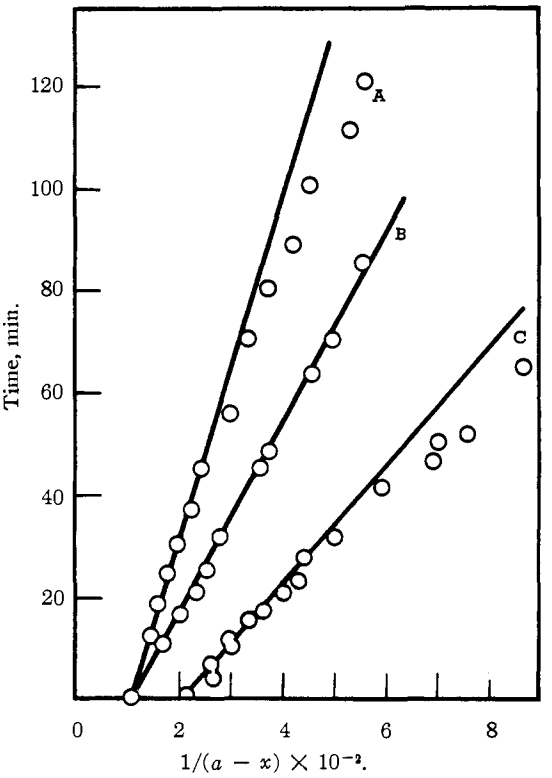

Fig. 1.—Alkaline hydrolysis at 25.04° in aqueous methanol: A, ethyl β-phenylpropionate, 30% methanol; B, methyl β-phenylpropionate, 20% methanol; C, benzoyl-L-phenylalanine ethyl ester, 50% methanol.

Carbonyl oxygen exchange experiments were performed during the alkaline hydrolysis of these three esters. In each case, oxygen exchange was found between the carbonyl oxygen and the solvent. The rate of the oxygen exchange reaction was determined according to the formulation of Bender[14] for benzoate esters

$$\log x/x_0 = k_e/k_h \log (E)/(E_0)$$

where x is the excess atom fraction of oxygen-18 in the ester whose concentration is (E) and where k_e and k_h are the respective rate constants of oxygen exchange and hydrolysis. The results of the oxygen exchange experiments for these three esters are shown in Fig. 2. The values of k_h/k_e obtained from

(14) M. L. Bender, THIS JOURNAL, 73, 1626 (1951).

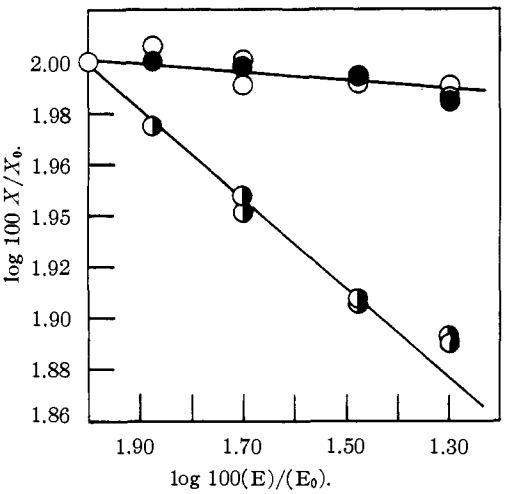

Fig. 2.—Carbonyl oxygen exchange *versus* extent of alkaline hydrolysis: ○, methyl β-phenylpropionate-*carbonyl*-O¹⁸; ●, ethyl β-phenylpropionate-*carbonyl*-O¹⁸; ◑, benzoyl-L-phenylalanine ethyl ester-*carbonyl*-O¹⁸.

the slopes of the lines are presented in Table II. It is interesting to note the large difference between the value of k_h/k_e for methyl and ethyl β-phenylpropionate and for benzoyl-L-phenylalanine ethyl ester. The value of 5.6 for k_h/k_e for the latter compound is comparable to those reported for ethyl, isopropyl and *t*-butyl benzoates.[14] No explanation can be offered for the relatively large value of k_h/k_e of 65 found for methyl and ethyl β-phenylpropionates. It should be pointed out that the values of k_h/k_e for the methyl and ethyl β-phenylpropionates are equivalent largely because the ethyl ester was converted to the methyl ester during the hydrolysis.

TABLE II

RATES OF ALKALINE HYDROLYSIS AND OXYGEN EXCHANGE OF SEVERAL ESTERS

Ester	$10^2 k_h$, l./mole sec.	k_h/k_e
Methyl β-phenylpropionate	9.48 ± 0.27[a]	65
Ethyl β-phenylpropionate	5.49 ± 0.52[b]	65
Benzoyl-L-phenylalanine ethyl ester	15.6 ± 1.1[c]	5.6

[a] 20% methanol–water. [b] 30% methanol–water. [c] 50% methanol–water.

The carbonyl oxygen exchange during the α-chymotrypsin-catalyzed hydrolysis of methyl β-phenylpropionate-*carbonyl*-O¹⁸ and of benzoyl-L-phenylalanine methyl and ethyl esters-*carbonyl*-O¹⁸ was studied. The exchange data for these esters are summarized in Table III.

The equivalence of the atom % O¹⁸ excess of the original ester, the ester recovered after partial hydrolysis, and the ester recovered from a blank experiment indicates that in these three cases no carbonyl oxygen exchange accompanies the α-chymotrypsin-catalyzed hydrolysis of an ester. It is to be noted that oxygen exchange was observed during the alkaline hydrolysis of several esters, while no such exchange occurred during the α-chymotrypsin-catalyzed hydrolysis of corresponding esters.

TABLE III

CARBONYL OXYGEN EXCHANGE DURING α-CHYMOTRYPSIN-CATALYZED ESTER HYDROLYSIS

Ester	Hydrolysis, %	Atom % O^{18} excess
Methyl	0	0.788
β-Phenylpropionate[a,d]	50	.78
	Blank[c]	.78
	50	.730[h]
	Blank	.730[h]
	50	.786[e]
	Blank	.783[e]
Benzoyl-L-phenylalanine	0	.507
Methyl ester[b,f]	50	.507
Benzoyl-L-phenylalanine	0	.507
Ethyl ester[b,g]	Blank	.507
	50	.502
	80	.504
	80	.500

[a] Atom % O^{18} calculated from equation 1. [b] Atom % O^{18} calculated from equation 2. [c] The blank refers to an experiment in which all components were present except enzyme. [d] 10% methanol, *p*H 7.8, buffer 0.015 *M* phosphate, 9.5 mg. enzyme/ml., 0.061 *M* ester. [e] 10% methanol, *p*H 7.8, buffer 0.03 *M* tris-(hydroxymethyl)-aminomethane, 9×10^{-4} *M* calcium chloride, 1.0 mg. enzyme/ml., 0.0061 *M* ester. [f] 30% methanol, *p*H 7.8, buffer 0.015 *M* phosphate, 1×10^{-2} mg. enzyme/ml., 0.0049 *M* ester. [g] 30% methanol, *p*H 7.8, buffer 0.019 phosphate, 5×10^{-3} mg. enzyme/ml., 0.002 *M* ester. [h] Apparently a constant error in the run as well as in the blank.

Alcoholysis during Alkaline and α-Chymotrypsin-catalyzed Hydrolysis.—In any ester hydrolysis in which a mixed water–alcohol solvent is used, there exists the possibility of alcoholysis occurring simultaneously with the hydrolysis. If the alcohol of the solvent is different from the alcoholic portion of the ester, one may readily observe this alcoholysis because of the different physical and chemical properties, of the newly formed ester. If the alcohol of the solvent is the same as the alcoholic portion of the ester, the alcoholysis should likewise occur and one may observe this virtual (or exchange) reaction by the use of oxygen-18. Both of these types of alcoholysis have been observed to accompany the hydrolytic reactions studied here.

It was noted previously that the rate constant for the alkaline hydrolysis of ethyl β-phenylpropionate and benzoyl-L-phenylalanine ethyl ester in aqueous methanol increased with the extent of hydrolysis. Figure 1 indicates that the initial slope of ethyl β-phenylpropionate is different from that of the corresponding methyl ester, but the slope near completion of the reaction is virtually identical with that of the methyl ester; clearly, the ethyl ester had been converted to the methyl ester during the course of the hydrolysis.

The melting points of the recovered benzoyl-L-phenylalanine ethyl ester after various extents of alkaline hydrolysis in aqueous methanol are given in Table IV, together with the melting points of mixtures of the methyl and ethyl esters of benzoyl-L-phenylalanine.

It is seen that the melting points of the recovered ester decrease with the extent of hydrolysis, and approach that of the methyl ester. The melting points of the recovered esters in conjunction with the melting points of the mixtures suggest that the recovered ester is a mixture of benzoyl-L-phenylalanine methyl and ethyl esters. The infrared spectra in Fig. 3 conclusively show that methanolysis has occurred. The spectrum of the ethyl ester of benzoyl-L-phenylalanine displays two absorption bands in the region of 1340 cm.$^{-1}$ while the methyl ester has only one band in this region at 1365 cm.$^{-1}$. Furthermore the spectrum of the ethyl ester has a shoulder at 1450 cm.$^{-1}$ whereas the methyl ester has a weak peak at this wave number. The spectra of the recovered esters are seen to change continuously from the ethyl ester to the methyl ester as the hydrolysis proceeds. In fact, the spectrum of the ester recovered after 80% hydrolysis is identical with that of the methyl ester.

TABLE IV

RECOVERED BENZOYL-L-PHENYLALANINE ETHYL ESTER AFTER VARIOUS EXTENTS OF HYDROLYSIS

Substance	Melting point, °C. Alkaline hydrolysis[a]	Melting point, °C. α-Chymotrypsin-catalyzed hydrolysis[b]
Pure ethyl ester	103.2–103.7	103.2–103.7[c]
Recovered after 25% hydrolysis	95 – 98	
Recovered after 50% hydrolysis	86 – 88	80.5– 81
Recovered after 70% hydrolysis	75 – 77	
Recovered after 80% hydrolysis	80 – 80.5	80.5– 81
Pure methyl ester	81 – 81.3	81 – 81.3
33% methyl–67% ethyl ester	88 – 97	
50% methyl–50% ethyl ester	79.5– 80.5	
67% methyl–33% ethyl ester	81 – 81.5	
Mixture of methyl ester and ester recovd. after 80% hydrolysis	79.5– 80.5	

[a] 50% aqueous methanol. [b] 30% aqueous methanol, *p*H 7.8, buffer 0.019 *M* phosphate, 5×10^{-3} mg. enzyme/ml., 0.002 *M* ester. [c] This value is for a blank in the absence of enzyme and corresponds to a virtual hydrolysis of 80%.

Benzoyl-L-phenylalanine ethyl ester was also hydrolyzed by α-chymotrypsin in 30% methanol–water solution. The melting points of the esters recovered after 50% and 80% hydrolysis are also listed in Table IV. The melting points indicate that by 50% hydrolysis considerable methanolysis had occurred. This is confirmed by the infrared spectra shown in Fig. 3. The spectra of the recovered esters after 50 and 80% hydrolysis are almost identical with that of the methyl ester, indicating that alcoholysis was complete at 50% hydrolysis.

Methyl β-phenylpropionate-*alkoxyl*-O^{18} was hydrolyzed by α-chymotrypsin in 15% aqueous methanol. The oxygen-18 contents of the recovered esters are listed in Table V. At 50% hydrolysis, the oxygen-18 content of the ester had disappeared, indicating methanolysis. From the alcoholysis reactions of these two esters it may be suggested that α-chymotrypsin generally catalyzes alcoholysis and that enzymatic alcoholysis is considerably faster than enzymatic hydrolysis, since the former is apparently complete when the latter is only 50% complete. Quantitative measurements of this competition are currently in progress in this Laboratory.

Mode of Fission in the α-Chymotrypsin-catalyzed Hydrolysis of Methyl β-Phenylpropionate.—Isotopic oxygen analysis of the β-phenylpropionic acid produced from the α-chymotrypsin-catalyzed hydrolysis of methyl β-phenylpropionate-*alkoxyl*-O^{18} indicated that the acid contains no excess isotopic oxygen. The data are given in Table V.

TABLE V

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF METHYL β-PHENYLPROPIONATE-*alkoxyl*-O^{18}[a]

Substance	Atom % O^{18} excess
Original ester	0.164
Blank ester[b]	.158
Ester recovd. after 50% hydrolysis	.008
Ester recovd. after 50% hydrolysis	.007
Acid recovd. after 50% hydrolysis	.00[c]
Acid recovd. after 50% hydrolysis	.00[c]

[a] 15% methanol, *p*H 7.8, 0.033 *M* phosphate buffer, 9 mg. enzyme/ml., 0.068 *M* ester. [b] Ester recovered after an interval corresponding to 50% hydrolysis in the absence of enzyme. [c] α-Chymotrypsin does not catalyze the oxygen exchange of β-phenylpropionic acid.[7]

These results indicate that the hydrolysis occurs with acyl–oxygen fission, although the concurrent methanolysis introduces some ambiguity into the argument. However, the methanolysis reaction, *per se*, supports the conclusion of acyl–oxygen fission. Acyl–oxygen fission also has been noted in ester hydrolysis by acetylcholinesterase.[15]

Discussion

α-Chymotrypsin has been found to catalyze the hydrolysis of methyl β-phenylpropionate by means of acyl–oxygen fission, the usual type of fission occurring in non-enzymatic hydrolysis.[5] However, the α-chymotrypsin-catalyzed hydrolysis of methyl β-phenylpropionate and benzoyl-L-phenylalanine methyl and ethyl esters differs from non-enzymatic (alkaline) hydrolysis of esters in that the latter reactions occurs with carbonyl oxygen exchange while no such exchange accompanies the enzymatic hydrolysis. One may, therefore, immediately rule out a symmetrical addition intermediate in this hydrolysis as was postulated for the non-enzymatic hydrolysis of esters.[14]

The lack of carbonyl oxygen exchange during the enzyme-catalyzed hydrolysis can be explained by a double displacement reaction,[16] a single displacement mechanism[16] in which the carbonyl oxygen atom is bound to the enzyme or to a concerted mechanism.[17,18] In the double displacement mechanism, the enzyme is assumed to make a nucleophilic attack upon the carbonyl carbon atom of the ester, displacing the alkoxyl group resulting in a typical acyl–oxygen fission. An unstable acyl–enzyme intermediate is formed in this process which is readily decomposed by an attack of a water molecule upon the carbonyl carbon atom of the acyl–enzyme intermediate. No exchange of the carbonyl oxygen of the ester can occur, since at no time in this process is the carbonyl oxygen equivalent to any other oxygen atom.

The lack of oxygen exchange of the ester can also be accounted for by assuming that the ester is bound to the enzyme through the carbonyl oxygen atom. A single displacement mechanism involving addition of a molecule of water to the carbonyl group of the ester would then produce an intermediate in which the labeled oxygen atom is not equivalent to the oxygen introduced by the water molecule. This clearly would exclude the possibility of oxygen exchange.

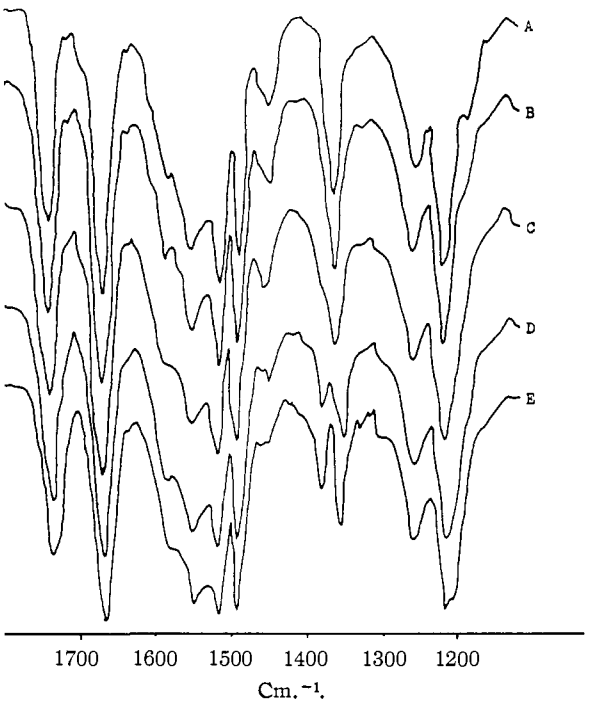

Fig. 3.—Infrared spectra of recovered benzoyl-L-phenylalanine ethyl ester after various extents of hydrolysis: A, benzoyl-L-phenylalanine methyl ester; B, benzoyl-L-phenylalanine ethyl ester recovered after 50 and 80% α-chymotrypsin-catalyzed hydrolysis and after 80% alkaline hydrolysis; C, benzoyl-L-phenylalanine ethyl ester recovered after 50% alkaline hydrolysis; D, benzoyl-L-phenylalanine ethyl ester recovered after 25% alkaline hydrolysis; E, benzoyl-L-phenylalanine ethyl ester.

A concerted process involving the enzyme as a bifunctional catalyst can also account for the lack of carbonyl oxygen exchange during the hydrolysis of esters. Neither the mechanism of Swain[18] nor that of Laidler[17] can lead to oxygen exchange because of the non-equivalence of the oxygen atoms in the concerted processes.

α-Chymotrypsin has been shown to catalyze a variety of exchange reactions, both in this study and in other studies. In addition to catalyzing the exchange of methanol with methyl β-phenylpropionate and benzoyl-L-phenylalanine ethyl ester demonstrated here, α-chymotrypsin also catalyzes the exchange of glycinamide with benzoyl-L-tyrosinamide[19] and the oxygen exchange of carboxylic acids.[7] From a consideration of several exchange reactions catalyzed by α-chymotrypsin, Koshland has suggested that the double displacement mechanism, in which an acyl–enzyme intermediate is formed, is the most probable mode of action of the enzyme.[16] For these exchange reactions to occur by the single (direct) displacement

(15) S. Stein and D. E. Koshland, Jr., *Arch. Biochem. Biophys.*, **45**, 467 (1953).

(16) D. E. Koshland, Jr., in "Mechanism of Enzyme Action," edited by McElroy and Glass, Johns Hopkins Univ. Press, Baltimore, Md., 1954, pp. 612–615.

(17) K. Laidler, "Introduction to the Chemistry of Enzymes," McGraw-Hill Book Co., Inc., New York, N.Y., 1954, p. 104.

(18) C. G. Swain and J. F. Brown, THIS JOURNAL, **74**, 2538 (1952).

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reaction on the enzyme surface, either glycinamide or methanol would be required to occupy a site normally occupied by the water molecule in the hydrolysis reaction. Because of the specificity exhibited by the enzyme the latter requirement appears too stringent and would not likely be fulfilled. A more reasonable explanation would be that a relatively stable acyl–enzyme intermediate is formed which is capable of reacting with nonbonded water, methanol or glycinamide molecules. One can explain the concurrent methanolysis and hydrolysis reactions by a competition of the nucleophiles, water and methanol for the acyl–enzyme intermediate. From the results presented here, it appears that methanol is a better nucleophile than water. Presumably in the transamination and transpeptidation reactions,[19] the same kind of competition exists. The hypothesis that the hydrolysis, methanolysis, transamination and transpeptidation reactions occur *via* an acyl–enzyme intermediate suggests that the distinction between the hydrolysis and transfer properties of enzymes may be overdrawn.

The evidence presented in this investigation in conjunction with that of studies mentioned above and with investigations of *p*H[20,21] and of diisopropylfluorophosphate inactivation[22] is consistent with a double displacement mechanism for the hydrolytic action of α-chymotrypsin. A similar mechanism has been described by Wilson[23] for the action of acetylcholinesterase. In contrast to the mechanism of Wilson, it is suggested that the enzyme–substrate complex, defined by the Michaelis–Menten constant, consists of adsorbed substrate on the enzyme in which the ester linkage has not been altered by the enzyme; that is, binding involves those parts of the molecule other than the ester group. We would then interpret Wilson's intermediates as steps in the activation process, involved in the rate constant, k_3. These intermediates, all short-lived, include the product of addition of a nucleophilic agent to the carbonyl carbon atom (a tetrahedral intermediate), the acyl–enzyme intermediate and the product of addition of a molecule of water to the acyl–enzyme intermediate (a second tetrahedral intermediate). The instability of the two tetrahedral intermediates is indicated by analogy with such compounds in non-enzymatic hydrolysis.[14] The instability of the acyl–enzyme intermediate is indicated by analogy with the instability of acetylimidazole.[24] Reversible formation of an enzyme–substrate complex, which is indicated in several α-chymotrypsin systems,[7] would not be possible with such unstable intermediates[14] but must rather involve an adsorbed substrate with an intact ester linkage. While this double displacement mechanism ininvolving an acyl–enzyme intermediate is consistent with all presently known facts, it clearly must await further definitive proof.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, ILLINOIS INSTITUTE OF TECHNOLOGY]

The Kinetics of the α-Chymotrypsin-catalyzed Oxygen Exchange of Carboxylic Acids[1]

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A study was made of the kinetics of the α-chymotrypsin-catalyzed oxygen exchange between the solvent, water, and the following acids which were labeled with oxygen-18 in the carboxyl group: benzoyl-L-phenylalanine, acetyl-L-tryptophan, benzoyl-D-phenylalanine and β-phenylpropionic acid. It was found that the first two acids underwent oxygen exchange with the solvent, whereas the last two did not. A symmetrical mechanism is proposed for the oxygen exchange. The values of K_O, the Michaelis constant of the oxygen exchange, and k', the composite rate constant of this reaction, were determined from simultaneous solution of equations of the type: $2.30 \log(x_o - 0.204)/(x - 0.204) = k'(E)_o t/(K_O + (S))$. Assuming negligible kinetic isotope effects, it is demonstrated that K_O is equal to k_2/k_1, an equilibrium constant. The values of K_O determined for the oxygen exchange of benzoyl-L-phenylalanine and of acetyl-L-tryptophan are in good agreement with the values of K_I determined for these substances when used as competitive (product) inhibitors. The equivalence of K_O and K_I suggests that K_I for product inhibition is an equilibrium constant in these cases. The similarity of enzymatic oxygen exchange and enzymatic hydrolysis with respect to mechanism and kinetics is pointed out. This similarity suggests that in the determination of the kinetics of hydrolytic reactions, the Michaelis constants may be approximately equilibrium constants and the k_3's may be composite rate constants. This conclusion, in conjunction with other data, suggests that the Michaelis constants for α-chymotrypsin–amide systems are equilibrium constants.

Introduction

Many of the carboxylic acids which are products of hydrolysis of α-chymotrypsin-catalyzed reactions are competitive inhibitors of this enzyme.[3] That is, they compete with the substrate for the active site on the enzyme. However, it has been shown that α-chymotrypsin catalyzes the exchange of oxygen atoms between the solvent and the car-

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(2) From the Ph.D. thesis of K.C.K.

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