Reaction of Papain with Ethyl [*carbonyl*-^{18}O]Hippurate*

Jack F. Kirsch† and Ephraim Katchalski

ABSTRACT: The rates of hydrolysis and oxygen-18 exchange of ethyl [*carbonyl*-^{18}O]hippurate were measured in both enzymatic (papain-catalyzed) and nonenzymatic (hydroxide ion–promoted) systems. The ratio of the rate constants for alkaline hydrolysis and oxygen-18 exchange, k_h/k_e, is equal to 14 in alkali but no exchange could be detected in the enzymatic system (i.e., $k_h/k_e >$ 80). The result is consistent with an enzymatic route of hydrolysis passing through either an acyl enzyme intermediate or through an enzyme-substrate complex so sterically hindered as to preclude oxygen-18 exchange. The data exclude intermediates or transition states identical to those formed during nonenzymatic hydrolysis. The papain-catalyzed hydrolysis of unlabeled ethyl hippurate was studied as a function of *p*H over the range *p*H 4.2–8.7. The K_m is constant within experimental error between *p*H 5.2 and 7.2, having a value of 0.018 M, and increases outside these limits. The rate constant for the product-forming step, k_{+2}, shows little variation from *p*H 4.2 to 8.7, having an average value of 3.3 sec^{-1}. This figure is much larger than the corresponding rate constant of 0.64 sec^{-1} determined by A. Stockell and E. L. Smith (*J. Biol. Chem. 227*, 1 [1957]) for hippurylamide, and proves that the latter constant cannot represent the rate-determining step for the deacylation of an hippuryl enzyme.

Smith and his colleagues (Smith, 1958; Smith and Kimmel, 1960) have proposed a detailed mechanism for the reactions of papain with ester and amide substrates which was based on the results of extensive kinetic and chemical investigations. An essential aspect of their proposal involves the acylation of the enzyme by the substrate at an active thiol site. Such a mechanism of hydrolysis does not allow the simultaneous equilibration of the carbonyl oxygen atom of the substrate with solvent. The usual nonenzymatic hydrolytic pathway, on the other hand, is generally accompanied by equilibration at this position (Bender, 1951). Ethyl [*carbonyl*-^{18}O]hippurate was synthesized, and the rates of hydroxide ion–promoted and papain-catalyzed hydrolysis and oxygen exchange reactions were measured in order to test the acyl enzyme hypothesis. A study similar to this one has been made by Bender and Kemp (1957) on chymotrypsin, a proteolytic enzyme not having a thiol group at its active center.

The Michaelis constant, K_m, and maximum velocity, V_{max}, were determined as functions of *p*H for HEE,[1] and the mechanistic significance of the rate constant for product formation, k_{+2}, obtained from these measurements is discussed.

Experimental

Materials. Benzoylarginine ethyl ester hydrochloride was prepared by Mr. Israel Jacobson; mp 128–130°, reported 129.5–131° (Kimmel and Smith, 1958). The preparation of triethylamine has been described previously (Kirsch and Jencks, 1964a). Oxygen-18-labeled water (5.5% ^{18}O) was obtained from Yeda Research and Development Co., Ltd., Rehovoth, Israel. Ordinary water was glass distilled from alkaline $KMnO_4$, and for use in certain experiments was stored in a CO_2-free atmosphere. Ethanol was redistilled. Papain (twice recrystallized) was purchased from Worthington Biochemical Corporation.

[*carboxyl*-^{18}O]Hippuric acid. A solution of 11 g (0.1 mole) glycine hydrochloride in 18 ml $H_2{}^{18}O$ was heated 3 days in a sealed ampule at 100°. After partial removal of the solvent the solution was made basic and the glycine was benzoylated according to the procedure of Greenstein and Winitz (1961). A total of 13 g (75% yield, 3.3% ^{18}O) was obtained after crystallization from ethanol.

[*carbonyl*-^{18}O]HEE. A sample of 9 g [*carboxyl*-^{18}O]-hippuric acid was suspended in 90 ml absolute ethanol at 0° and 9 ml thionyl chloride was slowly added. After 2 days at room temperature the solvent was removed to give an oil which was taken up in methylene chloride, washed successively with Na_2CO_3 solution and H_2O, and dried over Na_2SO_4. A yield of 8.1 g. (81%) was obtained after removal of the solvent and crystallization from 20% methanol-water, mp 60–61°, lit. 60.5° (Curtius, 1902); 1.1% ^{18}O.

Rate Measurements. The rate of alkaline hydrolysis of HEE was studied in 50% ethanol-water at 25 ± 0.05° and ionic strength 0.1 maintained with KCl. Reactions were initiated by adding a small volume of a

* From the Weizmann Institute of Science, Rehovoth, Israel. *Received December 18, 1964.*

† Fellow of The Helen Hay Whitney Foundation. Inquiries are to be made to J.F.K. at the Department of Biochemistry, University of California, Berkeley, Calif. 94720.

[1] The abbreviations used are: HEE, ethyl hippurate; BAEE, benzoylarginine ethyl ester; and BAA, benzoylargininamide.

concentrated solution of ester in absolute alcohol to the remainder of the reaction mixture to give a final ester concentration of 0.02 M. At appropriate time intervals 0.2-ml aliquots were withdrawn and the remaining quantity of ester assayed by the alkaline hydroxylamine method (Lipmann and Tuttle, 1945; Hestrin, 1949). The concentrations of HEE were determined from a standard calibration curve. The rates of hydrolysis were measured at 0.02, 0.03, and 0.04 M initial concentrations of KOH. Second-order rate constants were determined from plots of log $[OH^-]/[HEE]$ against time when the initial hydroxide ion concentration was greater than the initial ester concentration, or from plots of 1/[HEE] versus time where the initial concentrations were equal (Frost and Pearson, 1961). The method used for the determination of the saponification rates of esters under pseudo-first-order conditions has been described previously (Kirsch and Jencks, 1964b).

The kinetics of carbonyl oxygen exchange as a function of extent of alkaline hydrolysis were investigated under conditions of initially equal ester and KOH concentrations. At suitable time intervals 0.2-ml aliquots were taken for hydroxylamine assays and larger volumes were quenched in a slight excess of HCl. The ester was recovered from these larger aliquots by addition of excess sodium bicarbonate and extraction with CH_2Cl_2. The solvent was removed and the HEE was crystallized twice from CCl_4–petroleum ether. The melting point of the recovered ester was always identical to that of the starting material.

The papain-catalyzed exchange reactions were studied at 25° in 0.1 M citrate buffers, *p*H 5.8, in the presence of 5×10^{-3} M mercaptoethanol. The papain concentration determined by Kjehldahl analysis was 8.7×10^{-6} M based on a nitrogen content of 16.1% and a molecular weight of 20,700 (Smith and Kimmel, 1960). The reaction mixture minus papain and HEE was temperature equilibrated and the enzyme was added 5 minutes before addition of the ester in order to permit complete activation. The rate of the hydrolysis reaction was monitored by the following modification of the hydroxylamine assay: Aliquots of 0.2 ml were pipetted into 1 ml of a mixture containing 1 volume of 4 M $NH_2OH{\cdot}HCl$ and 2 volumes 3.5 M sodium hydroxide. After a few seconds 4 ml of 10% $FeCl_3{\cdot}6\,H_2O$ in 0.7 N HCl plus 0.8 ml of 40% trichloroacetic acid were added. Filtration proved to be unnecessary with this low concentration of protein and the optical densities of the samples were read at 540 mμ.

The remaining HEE was recovered from larger aliquots taken after times corresponding to 0, 50, and 90% hydrolysis of the ester. The reaction was stopped by the addition of trichloroacetic acid, and the ester was recovered by the method of Bender and Kemp (1957) and crystallized as before.

Oxygen-18 Analysis. The oxygen in HEE was converted to carbon dioxide by the method of Rittenberg and Pontecorvo (1956). The CO_2 was analyzed by measuring the mass 44/mass 46 ratio in a Consolidated Model 21-401 mass spectrometer. The atom fraction oxygen-18, n, was calculated from the relationship

$$n = \frac{1}{2(R + 1/2)} \qquad (1)$$

where R is the mass 44/mass 46 ratio (Dostrovsky and Klein, 1952), and the atom fraction excess oxygen-18 (x, equation 3) is obtained by subtracting the atom fraction oxygen-18 in normal CO_2. Analyses made on duplicate samples generally agreed to within ±1%.

Enzyme Kinetics. Determinations of K_m and V_{max} were carried out with the aid of a Radiometer TTTlc automatic titrator. The electrodes were standardized at the temperature of the experiments, 38°, with Fisher Scientific Co. reference buffers. Reactions were run in a total volume of 10 ml containing 0.005 M mercaptoethanol, 5×10^{-4} M EDTA, and 7.5×10^{-7} M papain. Ionic strength was maintained at 0.3 with KCl. The rates of reaction of HEE with papain were measured at six initial concentrations of ester, 0.0025, 0.005, 0.01, 0.015, 0.025, and 0.040 M. After temperature equilibration, reactions were initiated by the addition of papain and the desired *p*H was maintained by the automatic addition of 0.1 N KOH. The total volume of KOH added never exceeded 0.2 ml during the course of the experiment, and in all experiments less than 2% of the initial ester concentration was consumed. The *p*H of the reaction mixture was usually initially set between 0.1 and 0.3 *p*H unit above that at which the reaction was to be followed. This permitted measurement of the rate of hydrolysis at the desired *p*H after the enzyme was fully activated (1–2 minutes after addition of enzyme). It was noted in preliminary experiments that relatively high rates of reaction were occasionally observed before the addition of enzyme. This was apparently owing to the fact that enzyme from the previous run was adsorbed onto the electrodes and the walls of the reaction vessel. These blank rates could be lowered to insignificant levels by washing the reaction vessel and electrodes with 0.1 N HCl after each kinetic determination. At the higher *p*H values, 8.2 and 8.7, the rates of alkaline hydrolysis of HEE were not negligible in comparison to the rates of enzymatic hydrolysis, and the slopes of the lines plotted by the recorder in the absence of enzyme had to be subtracted from the total slope after papain had been added. Less than 1 mole of KOH is consumed per mole of ester hydrolyzed at *p*H 4.2 because of the incomplete ionization of hippuric acid. The rates measured at this *p*H were corrected for this by the use of the Henderson-Hasselbach equation and the known pK_a of 3.6 for hippuric acid at ionic strength 0.3 (Josephson, 1933).

Initial rates, v_i, were determined from the slopes of the straight lines plotted by the recorder. Values of K_m and V_{max} were obtained from plots of $[S]/v_i$ versus [S] where [S] is substrate concentration. The apparent rate constants for the decomposition of the enzyme substrate complex were found from the relationship

$$k_{+2} = \frac{V_{max}}{[E]} \qquad (2)$$

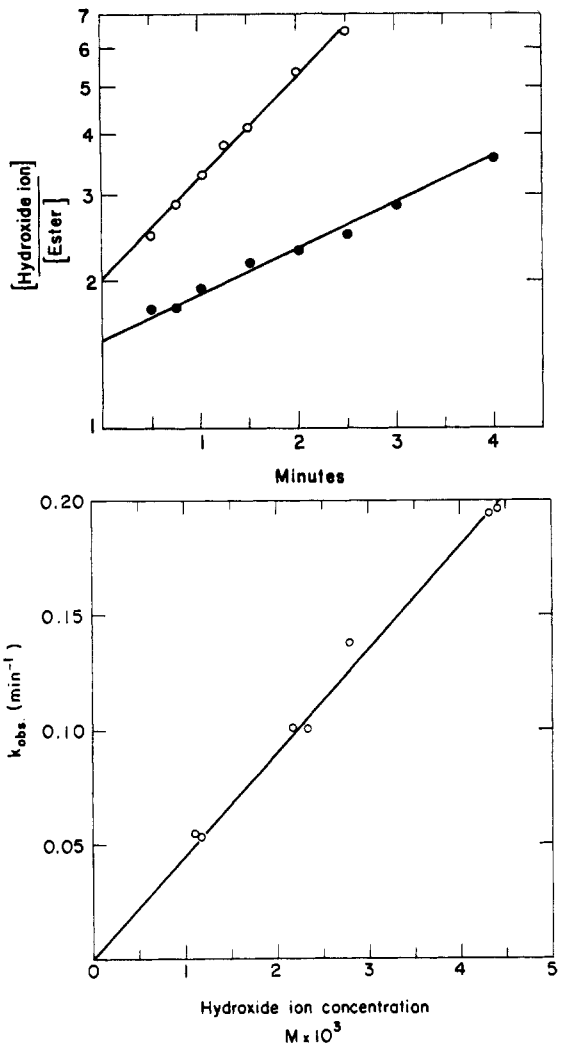

FIGURE 1: Alkaline hydrolysis of ethyl hippurate at 25° and 50% (v/v) aqueous ethanol and ionic strength 0.1 and in 0.1 M triethylamine buffers, ionic strength 1.0. (a, upper) Alkaline hydrolysis in ethanol. Initial ester concentration 0.02 M; (●), initial KOH 0.03 M; (○), initial KOH 0.04 M. (b, lower) Observed first-order rate constants for the hydrolysis of HEE in triethylamine buffers as a function of hydroxide ion concentration.

where [E] denotes total enzyme concentration. The values of K_m and k_{+2} so obtained have an estimated error of approximately ±5% between *p*H 5.2 and 7.2. On either side of this range, where the corrections discussed had to be applied, the estimated error increased proportionately to an approximate maximum of ±15% at *p*H 8.7.

Results

The second-order rate constant for the alkaline hydrolysis of HEE was determined under second-order conditions in 50% ethanol at three different initial concentrations of added potassium hydroxide corresponding to [KOH]/[ester] ratios of 2:1, 1.5:1, and 1:1. Data from two typical experiments are shown in Figure 1a. The value of the second-order rate constant thus obtained is given in Table I and may be compared with those determinations made under pseudo-first-order conditions in 2.5% ethanol-water using triethylamine buffers (Figure 1b and Table I). Buffer catalysis was shown to be negligible under these conditions (Table I, footnote *a*).

TABLE I: Rates of Alkaline Hydrolysis and Carbonyl Oxygen Exchange of HEE at 25°.

Solvent	No. of Determinations	k_{hyd} (M^{-1} min^{-1})	k_{hyd}/k_{ex}
2.5% Ethanol-water; ionic strength 1.0	7	45.9 ± 2.3[a]	
50% Ethanol-water (v/v); ionic strength 0.1	10	22.3 ± 4.2	14[b]

[a] Determined in 0.1 M triethylamine buffers. The same second-order rate constant was obtained within experimental error when the buffer concentration was halved. [b] The identical value was obtained in a duplicate experiment.

When [*carbonyl*-^{18}O]HEE was recovered from a partially saponified reaction mixture, the remaining ester was found to have a decreased oxygen-18 content which depended on the extent of hydrolysis. Quantitatively, the parallel between the rate of exchange and the rate of hydrolysis can be described by the relationship

$$\log \frac{x}{x_o} = \frac{k_e}{k_h} \log \frac{[E]}{[E_o]} \qquad (3)$$

where x corresponds to the per cent oxygen-18 excess in the ester of concentration [E], and the rate constants of the exchange and hydrolysis reactions are given by k_e and k_h, respectively (Bender, 1951) (Figure 2).

The ratio of rate constants k_h/k_e is equal to 14 for HEE when determined in 50% ethanol at 25°. This may be compared with the corresponding ratio of 5.6 for the only other *N*-acylamino acid ester in the literature, benzoyl-L-phenylalanine ethyl ester, determined at 25° in 50% methanol-water[2] (Bender and Kemp, 1957).

The papain-catalyzed hydrolysis of HEE followed pseudo-first-order kinetics (Figure 3) and large ali-

[2] The ratio 5.6 actually refers to a mixture of the methyl and ethyl esters because of an alcoholysis reaction as pointed out by Bender and Kemp (1957).

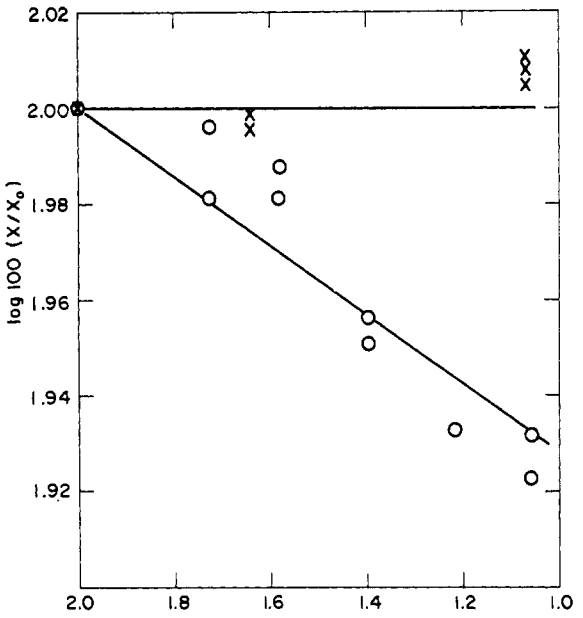

FIGURE 2: Extent of carbonyl oxygen exchange as a function of degree of reaction of HEE. (O), alkaline hydrolysis; (×), papain-catalyzed hydrolysis.

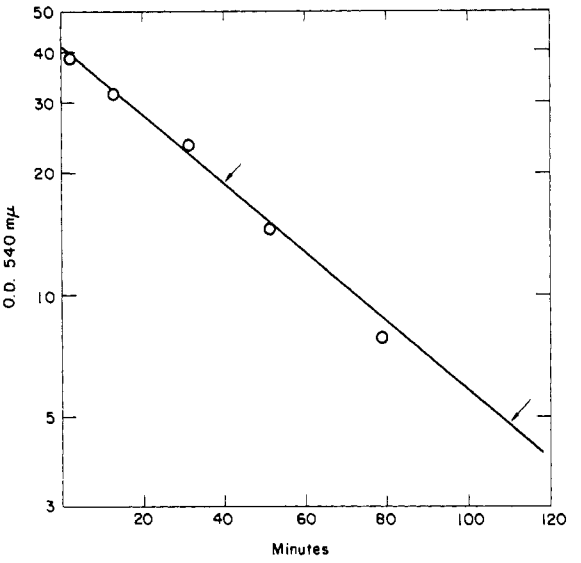

FIGURE 3: Rate of hydrolysis of initially 0.02 M HEE in 8.7×10^{-6} M papain at *p*H 5.8 and 25°. The arrows indicate the positions on the curve corresponding to the oxygen-18 analyses, (×) in Figure 2.

quots were taken from the reaction mixture at times corresponding to approximately 50 and 90% hydrolysis. The ester recovered from these aliquots did not differ within experimental error in oxygen-18 content from starting material (Figure 2).

Reactions of HEE with papain were also followed between 0.0025 and 0.04 M initial ester concentration at 38° over the *p*H range 4.2–8.7 with an automatic titrator set to maintain the desired *p*H without buffer. The results obeyed Michaelis-Menten kinetics over the entire range of *p*H as seen from the straight lines obtained from plots of $[S]/v_i$ versus [S] (Figure 4). In Figure 5, K_m and k_{+2} ($k_{+2} = V_m/[E]$) are plotted as functions of *p*H;[3] K_m is constant within experimental error from *p*H 5.2 to 7.2 where it has the value of 0.018 M, and increases beyond this range. The rate constant for the product-forming step, k_{+2}, shows little variation between *p*H 4.2 and 8.7, over which range it has an average value of 3.3 sec^{-1}. This rate constant is significantly greater than the corresponding one of 0.64 sec^{-1} for hippurylamide obtained by Stockell and Smith (1957). In order to ascertain whether this difference was a real one or the result of our using an enzyme of significantly higher activity, K_m and k_{+2} were determined for benzoylarginine ethyl ester at *p*H 6.2 and 25° under the conditions used by Smith and Parker (1958) except that 0.002 M mercaptoethanol plus 0.001 M EDTA were substituted for 0.0095 M 2,3-dimercaptopropanol. It was found that $K_m = 0.02$ M and $k_{+2} = 7.3$ sec^{-1}. These values compare with $K_m = 0.02$ M and $k_{+2} = 9$ sec^{-1} determined by the previous investigators, showing that the observation that k_{+2} for HEE is significantly larger than k_{+2} for hippurylamide is not owing to a difference in enzyme activity.

[3] After these experiments were completed a paper by Sluyterman (1964) appeared reporting the results of a study of the variation of K_m and k_{+2} for ethyl hippurate and papain at 40°. His results are in excellent agreement with those given here.

Discussion

Rate of Alkaline Hydrolysis. The rate constant for the alkaline hydrolysis of HEE in water is about seven times greater than that of ethyl acetate determined under the same conditions of temperature and ionic strength (Kirsch and Jencks, 1964b). The point for HEE lies satisfactorily close to a line of slope −1.2 drawn for a plot of log k_{OH^-} versus pK_a of the parent acid for a series of acyl-substituted ethyl acetates (data of Jencks and Carriuolo, 1961; Kirsch and Jencks, 1964b), demonstrating that this increased rate can be largely attributed to the electron withdrawal of the acyl moiety of hippuric acid.

Oxygen Exchange Concurrent with Alkaline Hydrolysis. The exchange of carbonyl oxygen-18 during the course of ester hydrolysis was first reported by Bender (1951) for the acid and alkaline hydrolysis of alkyl benzoates and has since been observed for the acid-catalyzed, alkaline, and neutral hydrolysis of a wide variety of esters (Bender, 1960; Bunton *et al.*, 1963) and benzamide (Bender and Ginger, 1955). The phenomenon has been rationalized in terms of a tetrahedral intermediate formed by a reversible addition of water or hydroxide ion to the acyl derivative. After a succession of proton transfers the intermediate can break down either to give products or to re-form the starting ester,

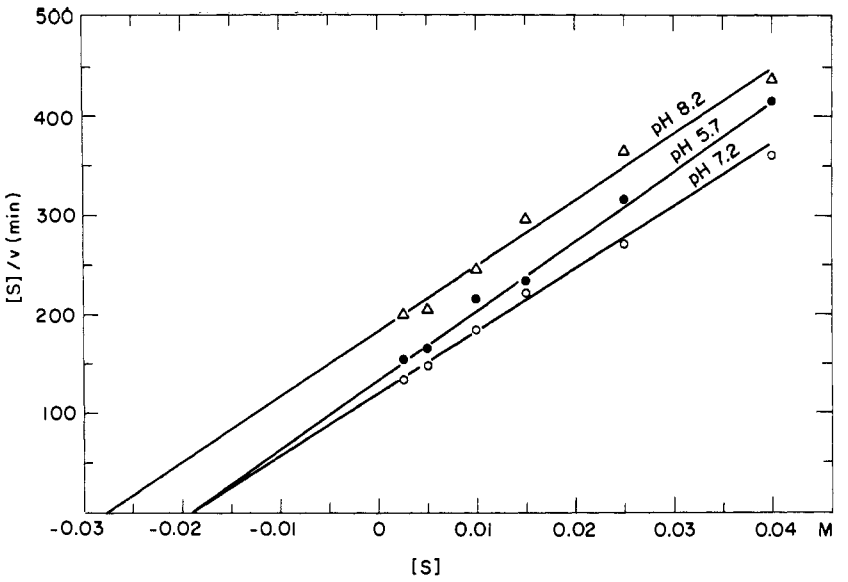

FIGURE 4: Typical plots of initial HEE concentration [S] divided by initial velocity (v) as a function of [S] at 38°, ionic strength 0.3, and 7.5×10^{-7} M papain.

but with a reduced carbonyl oxygen-18 content (Bender, 1951). It is possible to conceive of a mechanism for carbonyl oxygen exchange which does not involve a tetrahedral addition intermediate (Bunton, 1958); but regardless of mechanism, the fact of the occurrence of oxygen exchange requires that there be some equilibration of the acyl derivative with water before hydrolysis.

The ratio of the rate constant of hydrolysis to the rate constant of oxygen exchange, k_h/k_e, for ethyl esters varies widely. In general the ratio is lowered by increasing the acidity of the parent acid; thus k_h/k_e is 2.8 for methyl *p*-nitrobenzoate and 30 for methyl *p*-aminobenzoate (Bender and Thomas, 1961). The observed ratio for HEE, 14, is between these values.

The papain-catalyzed hydrolysis of HEE, in contrast to the hydroxide ion reaction, proceeds with no experimentally detectable exchange of carbonyl oxygen ($k_h/k_e > 80$), which means that at no point preceding the formation of the final products does the enzyme catalyze a quantitatively significant equilibration of substrate with water. The same observation was made by Bender and Kemp (1957) for the reactions of chymotrypsin with several ester substrates. They concluded that the results of their experiments are consistent with an acyl enzyme intermediate or with a direct displacement of the ethanol moiety of the ester by water if certain restrictions which exclude the formation of a free tetrahedral intermediate are operative. Such constraints would include binding of the ester by the enzyme at the carbonyl oxygen atom or a protonation of the leaving alkoxide group in concert with nucleophilic attack by water. The mechanism of hydrolysis of HEE by papain is apparently subject to the same restrictions.

Chymotrypsin (Sprinson and Rittenberg, 1951), pepsin (Sharon *et al.*, 1962), and papain (Grisaro and Sharon, 1964) all have been shown to catalyze oxygen exchange between water and virtual substrates, i.e., *N*-substituted amino acids. These experiments demonstrate acyl activation of the substrate, but do not distinguish between activation by formation of an intermediate acyl enzyme or by some other means such as concerted general acid-base catalysis.

It was originally proposed by Smith *et al.* (1955) that papain catalyzes the hydrolysis of esters and amides through the intermediate formation of a thiol ester from the acyl portion of the substrate and an active sulfhydryl group on the enzyme. The evidence that a sulfhydryl moiety is involved in the mechanism of action comes mainly from studies with activators and inhibitors (Smith and Kimmel, 1960; Sanner and Pihl, 1963); while that supporting intermediate acylation of the enzyme stems from two observations: (1) The rate constants for the decomposition of the enzyme-substrate complexes, k_{+2}, are identical for the substrates BAEE and BAA. Since amides are ordinarily hydrolyzed several orders of magnitude more slowly in nonenzymatic systems, k_{+2} is assumed to represent the rate constant for the decomposition of a common intermediate, the acyl enzyme. (2) Papain catalyzes transamidation as well as hydrolysis reactions (Fruton, 1950; Durell and Fruton, 1954). This observation is also readily accommodated by the postulated active derivative represented by an acyl enzyme. Both the foregoing observations are also consistent with explanations not involving acyl enzyme intermediates. An alternative mechanism leading to the identical k_{+2} for both the ester and amide substrates would be one in which the slow step in the product formation sequence occurs either before or after the breaking of the C—O or C—N

bond. One possible candidate for such a slow step might be a rate-determining conformational change. Transamidation reactions would be expected to occur in a facile manner with any nucleophile if the enzyme does not have a specific water site. Since nucleophilic reactions of ammonia (Bunnett and Davis, 1960; Jencks and Carriuolo, 1960) and other nitrogen nucleophiles (Anderson, 1962; Bruice and Benkovic, 1964; Kirsch and Jencks, 1964a) on acyl derivatives are general base catalyzed, there is no need to confine the substrate to one particular active configuration such as an acyl enzyme, as these other nucleophiles are capable of reacting with the original substrate in a direct catalyzed displacement. Although it is possible that the catalytic constants, k_{+2}, for BAA and BAEE represent deacylations, it is certain that the observed k_{+2} for hippurylamide does not, since the corresponding constant for HEE is nearly five times greater than that for hippurylamide (Figure 5; and Sluyterman, 1964). A similar result has been found in the case of ficin by Hammond and Gutfreund (1959), where the measured value of k_{+2} is the same for BAEE and BAA, but is 30-fold larger for HEE than for hippurylamide. It was suggested that these rate constants for the first three substrates represent deacylation steps, and that for hippurylamide, acylation. Bromelain, the third SH protease of this class to have received extensive kinetic study, differs from the first two in that k_{+2} for BAEE is 140-fold greater than for BAA (Inagami and Murachi, 1963).

Stockell and Smith (1957) measured the deuterium solvent isotope effect on k_{+2} for BAA and found $k_H/k_D = 1.0$. Such an observation implies that the rate-determining step in the complex of microconstants represented by k_{+2} did not involve the stretching of a solvent O—H bond in the transition state (e.g., Bunton and Shiner, 1961). While this observation was consistent with the working hypothesis involving the re-formation of a thiol ester originally present in the native enzyme (Smith, 1958), more recent evidence makes it appear likely that the active SH in the enzyme exists as such and is not bound as a thiol ester (Sanner and Pihl, 1963). The deacylation step, therefore, would almost certainly have to involve the breaking of a solvent OH bond. In the specific case of chymotrypsin it has been possible to study solvent isotope effects on the rates of deacylation of acyl chymotrypsins. The rates for these reactions are decreased more than 2-fold in D_2O (Bender *et al.*, 1961; Caplow and Jencks, 1962). If k_{+2} does, therefore, represent a deacylation step in the case of benzoylargininyl papain, then the mechanism must involve a distribution of protons in the transition state very different from that which obtains in the deacylation of acyl chymotrypsins. Bender and Brubacher (1964), however, have recently demonstrated the intermediate formation of cinnamoyl papain from *trans*-cinnamoylimidazole and have shown that the deacylation of this species is in fact markedly slower in D_2O.

Lowe and Williams (1964) have studied the reaction of methyl thionohippurate with papain and have observed the transient appearance of an intermediate having a spectrum resembling that of a dithioester. These results, along with those of Bender and Brubacher, provide the first direct evidence for the intermediate acylation of the enzyme sulfhydryl group by substrate. Additional experiments by one of the present authors have shown that k_{+2} is independent of the leaving group for several substituted phenyl esters of carbobenzoxyglycine.[4] These results are consistent with the intermediate formation of an acyl enzyme from these species. It is still to be determined whether the less reactive, more physiological substrates give rise to analogous intermediates.

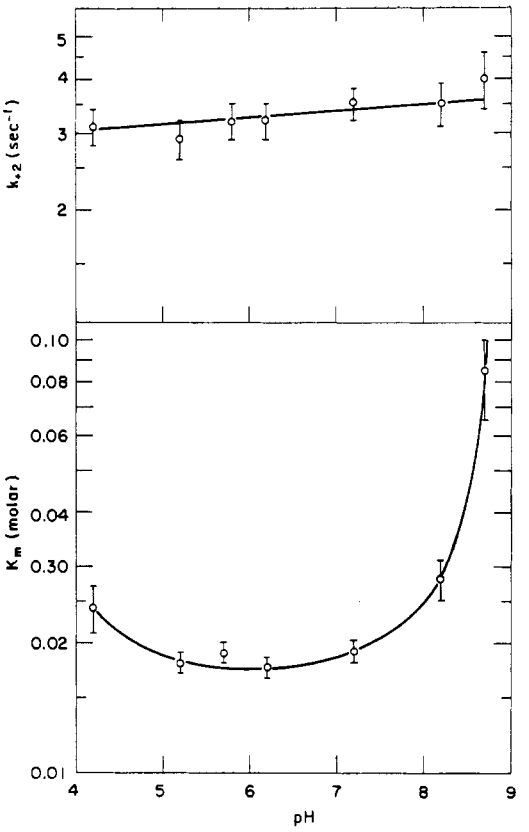

FIGURE 5: Logarithmic plots of K_m (lower curve) and k_{+2} (upper curve) as functions of *p*H, for the papain-catalyzed hydrolysis of HEE. Conditions are those given in the legend to Figure 4.

Acknowledgment

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[4] J. F. Kirsch and D. Kramer, unpublished results.

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