The Kinetics of the Papain-Catalyzed Hydrolysis of Esters of Carbobenzoxyglycine. Evidence for an Acyl-Enzyme Intermediate*

Jack F. Kirsch and Mona Igelström

ABSTRACT: Values of K_m and k_{cat} were determined for the papain-catalyzed hydrolyses of the *p*-nitrophenyl, *m*-nitrophenyl, *o*-nitrophenyl, and ethyl esters of carbobenzoxyglycine. The k_{cat} were very nearly identical and showed no correlation with substrate reactivity, as measured by the rate constants for the corresponding reactions of the esters with hydroxide ion and with mercaptoethanol. The rate constants for alkaline hydrolysis at 25° varied from 40.6 M^{-1} min^{-1} for the ethyl ester to 6900 M^{-1} min^{-1} for the *p*-nitrophenyl compound. The rates of reaction with mercaptoethanol were shown to be proportional to the concentration of the anionic species and varied from 19,000 M^{-1} min^{-1} for the *p*-nitrophenyl to 531 M^{-1} min^{-1} for the phenyl ester. A plot of log $1/K_m$ *vs.* log k_{OH^-} for these esters gave a straight line having a slope of 1.3, indicating that the measured K_m is not a pure binding constant but is in part a measure of the ease of nucleophilic displacement at the carbonyl carbon atom. These results support a mechanism of action of the enzyme in which the enzyme and substrate form a noncovalent complex in the first stage of reaction, which is followed by an acylation step with a rate constant dependent upon the reactivity of the substrate. The final reaction is the deacylation of the enzyme. A detailed mechanism is proposed in which the active sulfhydryl group acts as a nucleophile and a carboxyl moiety functions solely as a proton-transfer agent. The reaction of papain with the *p*-nitrophenyl ester provides a very sensitive and convenient assay.

Evidence collected largely in recent years has tended to support the hypothesis that the hydrolysis of simple substrates (*i.e.*, N-substituted amino acid esters and amides) by many proteolytic enzymes proceeds through acyl-enzyme intermediates formed from the acyl portion of the substrate and a reactive nucleophile on the enzyme (Bender and Kézdy, 1965). Several years ago, Smith (1958) proposed a mechanism of action for the enzyme, papain, in which the postulated acyl-enzyme intermediate was a thiol ester. Several pieces of experimental evidence supported this contention, but possibly the most striking argument for an acyl-enzyme intermediate was provided by the observation that V_{max} for benzoylarginine ethyl ester and benzoylargininamide were very nearly equal. Whitaker and Bender (1965) have recently redetermined the kinetic parameters for these two substrates and have found that V_{max} is about twofold greater for the ester than for the amide, but since amides ordinarily react several orders of magnitude more slowly than esters even in other enzymatic reactions (*e.g.*, Zerner *et al.*, 1964) these results still suggest a rate-determining step not involving or involving only in minor part the ease of nucleophilic displacement of the leaving group of the benzoylarginine substrates. This step is most reasonably the deacylation of a common acyl-enzyme intermediate. Other interpretations are possible, however, and it was decided to extend this data to a series of esters whose reactivity to nonenzymatic nucleophilic displacement could be measured. Accordingly, the kinetics of the papain-catalyzed hydrolyses of several esters of Zgly[1] were studied and correlated with the rates of the nonenzymatic reactions with ME and OH^-. Lowe and Williams (1965c) have recently reported the results of an independent similar study with esters of hippuric acid. Their experimental observations are in good agreement with those given in this communication.

Experimental Section

Materials. The phenyl esters of carbobenzoxyglycine were all prepared by the method used by Bodanszky and du Vigneaud (1962) for the preparation of the *p*-nitrophenyl compound. The ethyl ester was obtained in 79% yield by the esterification procedure previously described for hippuric acid (Kirsch and Katchalski, 1965). The substrates used in this study are listed in Table I together with their melting points and analyses for the two new compounds. They were all crystallized from ethanol or ethanol–water.

Papain (twice recrystallized) was purchased from Worthington Biochemical Corporation and cysteine

---

* From the Department of Biochemistry, University of California, Berkeley, California. *Received September 8, 1965.* Supported in part by a U. S. Public Health Service grant (GM 12278).

[1] The abbreviations used are: Zgly, carbobenzoxyglycine; ME, mercaptoethanol.

TABLE I: Esters of Carbobenzoxyglycine.

Ester	Mp (°C)	Lit
p-Nitrophenyl	127–8	124–5 (Iselin *et al.*, 1957)
m-Nitrophenyl	70–71	
calcd C 58.4; H 4.25; N 8.49		
found C 58.4; H 4.30; N 8.61[a]		
o-Nitrophenyl	68–69	
calcd C 58.4; H 4.25; N 8.49[a]		
found C 58.3; H 4.38; N 8.58		
Phenyl	67–68	67–68 (Karrer and Heynemann, 1948)
Ethyl	34–35	35.5–36.5 (Barkdoll and Ross, 1944)

[a] Microanalyses by A. Bernhardt, Max-Plank Institut fur Kohlenforschung, Mülheim (Ruhr), Germany.

was obtained from Calbiochem, Los Angeles, Calif. ME, acetonitrile, and triethylamine were redistilled. The amine was converted to the hydrochloride and stored as a 3.0 M aqueous solution at pH 2. Glass-distilled water was used in all kinetic studies and was CO_2-free when necessary.

Enzyme Kinetics. The standard incubation mixture for activating the enzyme contained 0.021 M phosphate buffer, pH 6.8, 0.001 M EDTA, and 3.5×10^{-4} M cysteine. Papain was added to a final concentration of 3.5×10^{-7} M and activation was carried out at 25°. Maximal enzyme activity was reached after 45 min and remained constant for at least 4 hr (see Results). The reactions of the substituted phenyl esters were followed by transferring 2.8 ml of the activated enzyme solution into a cuvet placed in a brass block thermostated at 25° in the cell compartment of a Zeiss PMQ II spectrophotometer. Sufficient acetonitrile was added to give a final concentration (after the addition of ester) of 6.7% and the reaction was initiated by adding a solution of the ester in acetonitrile with stirring to give a final volume in the cuvet of 3.0 ml. Absorbance readings were taken at 5-sec intervals and initial velocities determined from the slopes of the straight lines obtained when absorbance was plotted against time. Certain experiments employing low concentrations of ester required the use of cells with a 5.0 cm path length in order to obtain conveniently measurable changes in absorbance. In nearly all the experiments the linear relationship was maintained throughout a large fraction of the reaction because of the low values of K_m. The measurement of absorbance after the completion of the reaction provided the conversion factor required to express the results in moles/liter per minute.

The rates of reaction were also determined in the absence of enzyme and the slopes of these absorbance *vs.* time plots were subtracted from those determined with the enzyme included. The rate of the nonenzymatic reaction was negligible for the reactions of the phenyl ester, but varied from 2 to 4% of the enzymatic rate for the *m*-nitrophenyl, from 2 to 7% for the *p*-nitrophenyl, and between 15 and 35% for the *o*-nitrophenyl ester. The ratio of the blank rate to the enzymatic rate was lowest at the lower concentrations of substrate and increased as the substrate concentration was raised, reflecting the saturation curve of the enzyme.

The papain-catalyzed hydrolysis of Zgly ethyl ester was followed by automatic titration at constant pH employing an assembly built around a Radiometer-TTT 11 titrator, 25SE pH meter, SBR2c titragraph, and SBU1-syringe buret. Two methods were employed, the best results being obtained with the procedure described previously (Kirsch and Katchalski, 1965). These experiments were run in a water-jacketed reaction vessel at pH 6.8 and 25° in a total volume of 10 ml containing 0.005 M ME, 0.035 M KCl, 5×10^{-4} M EDTA, and 6% CH_3CN. The reaction was initiated by the addition of papain to a final concentration of 3.5×10^{-7} M. Somewhat less precise results were obtained under conditions similar to those in which the reactions of the substituted phenyl esters were studied. In these experiments the enzyme was activated for at least 45 min at 25° and pH 6.8–7.0 in the presence of the above concentrations of KCl and EDTA, with 3.5×10^{-4} M cysteine replacing the ME. A 9.4-ml aliquot of the activated enzyme was transferred to the reaction vessel, sufficient CH_3CN was added to give a final concentration of 6%, and the reaction initiated by the addition of ester. Initial velocities were obtained from the slopes of the lines drawn by the recorder after the pH reached 6.80.

The kinetic parameters K_m and V_{max} and their standard errors were evaluated with an IBM 7094 computer using a program (HYPER) described by Cleland (1963)[2] which is based on a weighted fit to the reciprocal form of the Michaelis–Menten equation (Wilkinson, 1961). The results were independently verified through the use of Program NLIN which was used to perform a nonlinear regression analysis on the hyperbolic form of the Michaelis–Menten equation using the unweighted raw data. The values of K_m and V_{max} calculated by the

---

[2] We are grateful to Professor Cleland for providing us with a copy of Program HYPER and to Dr. R. M. Baer of the University of California Computer Center for Program NLIN.

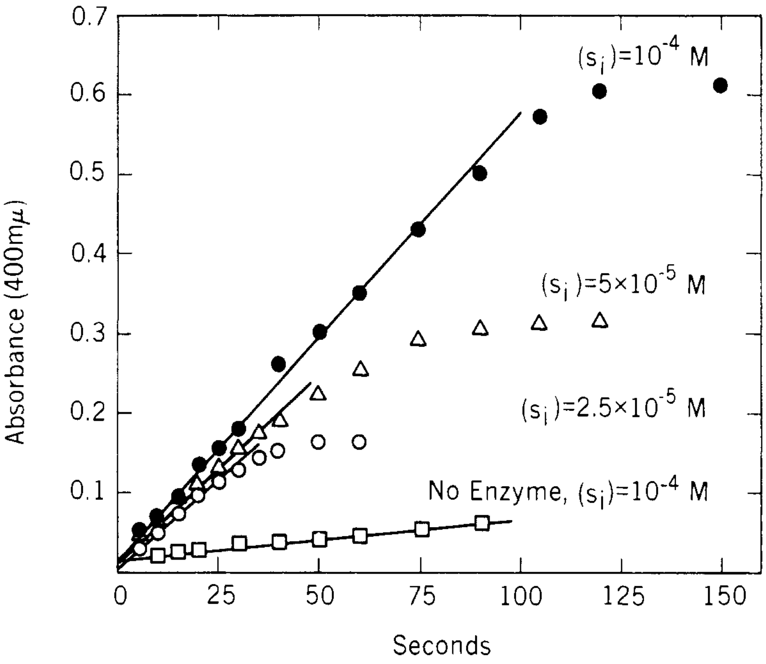


FIGURE 1: Papain-catalyzed hydrolysis of Zgly *p*-nitrophenyl ester at pH 6.8 and 25°. The reaction mixture contained 0.02 M sodium phosphate buffer, 0.001 M EDTA, 3.3 × 10^{-4} M cysteine, 6.7% (v/v) acetonitrile, and 3.3 × 10^{-7} M papain, initial substrate concentrations [s_i] as shown. The activation time before addition of substrate was at least 45 min and less than 4 hr (see Experimental Section and Figure 2).

two procedures were identical to two significant figures. Such close agreement was unexpected since HYPER assigns relative weights to the data whereas NLIN does not. Program NLIN does not provide standard errors. The rate constants, k_{cat}, were obtained from the relationship

$$k_{cat} = \frac{V_{max}}{[E]} \qquad (1)$$

Rates of Alkaline Hydrolysis. The rates of alkaline hydrolysis of the substituted phenyl esters were determined by the method described previously (Kirsch and Jencks, 1964b). Measurements of pH of the reaction mixture were taken before and after each kinetic run and the rate constants were calculated only for those runs where these determinations differed by less than 0.05 pH unit. The saponification rates of the ethyl ester were followed with the modified alkaline hydroxylamine assay (Kirsch and Jencks, 1964a).

Rates of Reaction with ME. The components of the reaction mixtures for these studies were incubated at 25° in glass-stoppered test tubes and the reactions initiated by the addition of small amounts of a concentrated solution of the ester in acetonitrile bringing the final volumes to 5.0 ml. The rates of appearance of the various phenols or phenolate ions were followed spectrophotometrically. Aqueous solutions of ME were prepared within 1 hr of use. Ionic strength was maintained at 0.3 with KCl. Second-order rate constants were obtained from the slopes of plots of k_{obsd} against ME concentration. The pK_a of ME was found by titration to be 9.51 ± 0.02 in 0.3 M KCl at 25°.

Results

Papain-Catalyzed Hydrolysis. The nitrophenyl ester of carbobenzoxyglycine like that of carbobenzoxytyrosine (Martin *et al.*, 1959) is an excellent substrate for papain. The reactions of the enzyme with this substrate at several different initial ester concentrations are shown in Figure 1. It is apparent from this figure that the initial rates of reaction are not very sensitive to substrate concentration, indicating that K_m is very low. The rate of appearance of nitrophenol in the absence of enzyme is appreciable and is dependent upon the concentrations of cysteine and of substrate. It was therefore necessary to use an activator concentration of 3.5 × 10^{-4} M cysteine, which is much lower than that customarily employed in studies of this enzyme [*e.g.*, Smith and Parker (1958) used 0.0095 M 2,3-dimercaptopropanol (BAL) in their studies on the hydrolysis of benzoylarginine ethyl ester and we previously found that 0.005 M ME is sufficient to completely activate papain in two min (Kirsch and Katchalski, 1965). Martin *et al.* (1959), however, were able to obtain complete activation with 8 × 10^{-5} M BAL]. The activation of the enzyme is not fast under the conditions used here, but is complete after 45 min, and constant activity is maintained for at least 4 hr (Figure 2), declining to about $^2/_3$ of the maxi-

TABLE II: The Kinetics of the Papain-Catalyzed Hydrolyses of Esters of Carbobenzoxyglycine at pH 6.8 and 25°.[a]

Ester	Concn (M × 10^4)	No. of Points	K_m[b] (M × 10^5)	k_{cat}[b,c] (sec^{-1})	Method
p-Nitrophenyl	0.08–1.0	21	0.93 ± 0.10	2.73 ± 0.08	Abs 400 mμ
m-Nitrophenyl	0.15–1.0	32	1.89 ± 0.17	2.18 ± 0.06	Abs 330 mμ
o-Nitrophenyl	0.60–3.2	11	15.2 ± 2.3	2.14 ± 0.15	Abs 415 mμ
Phenyl	0.9–5.0	14	10.7 ± 0.9	2.45 ± 0.06	Abs 270 mμ
Ethyl[d]	13–75	13	514 ± 74	1.96 ± 0.14	Autotitrator

[a] In 6.7% (v/v) acetonitrile, [E] = 3.3×10^{-7} M. [b] The kinetic parameters, K_m and V_{max}, and the associated standard errors, were determined from program HYPER (see Experimental Section). [c] $k_{cat} = V_{max}/[E]$. [d] 6.0% acetonitrile.

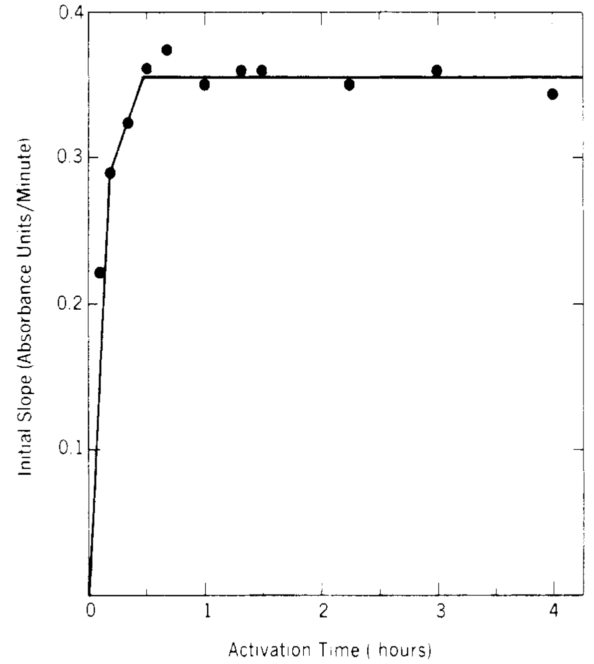


FIGURE 2: Activity of papain as a function of time of preincubation with activator. Assay conditions as in Figure 1 with 10^{-4} M Zgly *p*-nitrophenyl ester.

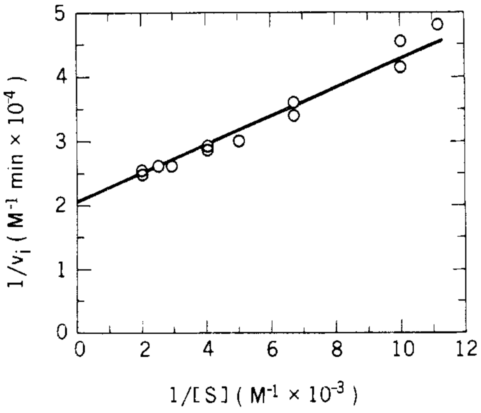


FIGURE 3: Lineweaver–Burk plot for the papain-catalyzed hydrolysis of Zgly phenyl ester. Assay conditions as in Figure 1. The points are experimental and the line represents the weighted least squares fit to the data obtained from program HYPER (see Experimental Section).

mum after 24 hr. Increasing the concentration of cysteine had no effect on the maximum velocity attained, but reduced the time required for complete activation. Less than 2% of the maximal initial velocity was obtained in the absence of activator. The activator concentration used in Figures 1 and 2 was chosen as a reasonable compromise allowing for both relatively short times of activation and small rates of nonenzymatic hydrolysis.

All reactions were studied in 6.0 or 6.7% (v/v) acetonitrile to facilitate the rate of solution of the esters. In order to determine whether or not this concentration of organic solvent has an important effect on the observed velocity, the rate of reaction of Zgly *p*-nitrophenyl ester with fully activated papain was studied at acetonitrile concentrations ranging from 0 to 50% (v/v). No significant variation in initial velocity was found with organic solvent concentrations of up to 20%, but the rates decreased at concentrations above this. The effect of organic solvent was studied more extensively by Stockell and Smith (1957) who observed a twofold increase in K_m and a 10% decrease in k_{cat} in the papain-catalyzed hydrolysis of benzoylargininamide when methanol concentration was increased from 0 to 20%.

The reactions of all the esters were studied at fixed enzyme concentrations and varying concentrations of ester. The data were plotted either as $1/v_i$ *vs.* $1/[s]$ or as $[s]/v_i$ *vs.* $[s]$ in order to search for regular deviations such as substrate or product activation or inhibition which might complicate the kinetics. No such phenomena were observed and the data for all the compounds gave good fits to straight lines when plotted according to either convention. A typical Lineweaver–Burk plot is shown in Figure 3. Values of K_m and k_{cat} and the associated standard errors as determined from computer

TABLE III: The Rates of Reaction of Esters of Carbobenzoxyglycine with Hydroxide Ion at 25° and Ionic Strength 1.0.[a]

Ester	Initial Ester Concn (M × 10^5)	Hydroxide Ion[b] (M × 10^4)	No. of Detmns	$k_{\rm OH}$[c] (M^{-1} min^{-1})	Method
p-Nitrophenyl	2.5	1.45–12.90	12	6900	Abs 400 mμ
m-Nitrophenyl	10	2.48–11.9	6	4050	Abs 390 mμ
o-Nitrophenyl	10	2.37–13.6	9	3680	Abs 400 mμ
Phenyl	10	2.36–14.6	12	728	Abs 287 mμ
Ethyl	50	18.8–70.0	11	40.6	Alk NH_2OH

[a] Determined in 0.05 or 0.1 M triethylamine buffers containing 1–2% acetonitrile. [b] Calculated from measured pH, activity coefficient 0.67, and $K_w = 10^{-14}$ (Kirsch and Jencks, 1964a). [c] $k_{\rm obsd}/[{\rm OH^-}]$. There was no appreciable buffer-catalyzed or neutral hydrolysis under these conditions as $k/_{\rm obsd}$ extrapolated to zero at $[{\rm OH^-}] = 0$ in all cases.

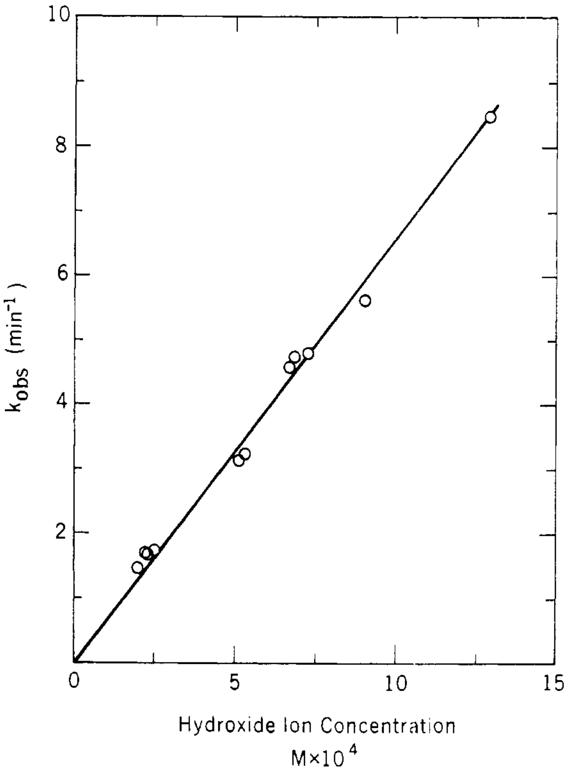


FIGURE 4: The observed first-order rate constants for the hydrolysis of Zgly *p*-nitrophenyl ester in 0.05 M triethylamine buffers as a function of hydroxide ion concentration at 25° and ionic strength 1.0.

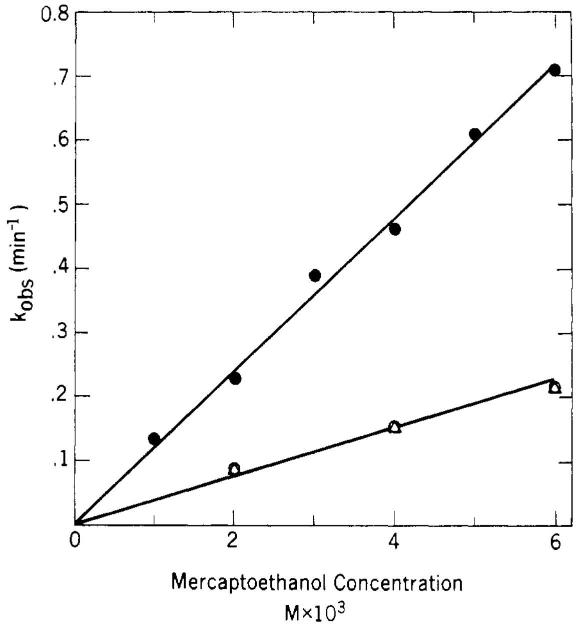


FIGURE 5: The rates of reaction of Zgly *o*-nitrophenyl ester with ME in 0.05 M phosphate buffers at 25° and ionic strength 0.3. (●) pH 7.49 ± 0.01; (Δ) (O) pH 7.00 ± 0.01.

analysis are collected in Table II. Since it was possible to make many measurements at substrate concentrations greater than K_m, $k_{\rm cat}$ was determined to a greater precision than K_m. The average value of the standard error for $k_{\rm cat}$ is *ca.* 4%, whereas that for K_m is *ca.* 12%.

Rates of Alkaline Hydrolysis. The rates of alkaline hydrolysis of the five esters of carbobenzoxyglycine were determined as a measure of the reactivity of these compounds. The observed rates of appearance of *p*-nitrophenol are shown as a function of [OH⁻] in Figure 4. The values of $k_{\rm obsd}$ extrapolate to zero at zero hydroxide ion concentration, demonstrating the absence of appreciable buffer or water catalysis under these conditions. In addition the neutral solvolysis of Zgly *p*-nitrophenyl ester was measured directly in 0.001, 0.0015, and 0.002 M HCl at 25° and ionic strength 1.0. This reaction is not acid catalyzed under these conditions and a rate constant of 5×10^{-6} (±25%) M^{-1} min^{-1} was obtained. The alkaline hydrolysis experiments are summarized in Table III.

Rates of Reaction with Mercaptoethanol. Since the

TABLE IV: The Rates of Reaction of Substituted Phenyl Esters of Carbobenzoxyglycine with Mercaptoethanol at 25° and Ionic Strength 0.3.[a]

Ester	Initial Ester Concn (M × 10^5)	Mercaptoethanol (M × 10^3)	pH	No. of Detmns	k_{RS^-}[b] (M^{-1} min^{-1})	k_{RS^-}[c] (M^{-1} min^{-1})	Method
p-Nitrophenyl	5.0	1.0–16.3	6.45 ± 0.02	9	17	19,500	Abs 400 mμ
	5.0	2.0– 6.0	7.00 ± 0.02	8	57	18,400	Abs 400 mμ
m-Nitrophenyl	10.0	2.0– 6.6	6.49 ± 0.01	8	10.5	11,000	Abs 330 mμ
o-Nitrophenyl	12.0	2.0– 6.0	7.00 ± 0.01	6	40.0	12,800	Abs 415 mμ
	12.0	2.0– 6.0	7.49 ± 0.01	6	119	12,400	Abs 415 mμ
Phenyl	30.0	4.0–12.0	7.00 ± 0.02	8	1.64	531	Abs 270 mμ

[a] Reactions measured in 0.05 M phosphate buffers and 0.5–2% acetonitrile. Doubling the buffer conception at constant ionic strength did not affect k_{obsd}. [b] k_{obsd}/[ME] total. Under these conditions $k/_{\text{obsd}}$ extrapolated to the origin when [ME] = 0. [c] For the reactive (anionic) species, calculated from the measured pH and the Henderson–Hasselbach equation using pK_a = 9.51 for ME which was determined at 25° and ionic strength 0.3.

most probable acyl-enzyme intermediate is a thiol ester (Smith, 1958; Lowe and Williams, 1964, 1965a; Bender and Brubacher, 1964), it was of interest to determine the relative susceptibility of these esters to nucleophilic attack by mercaptans. The rates of reaction of Zgly *o*-nitrophenyl ester with ME are shown at two different pH values in Figure 5. The rates increase with increasing pH at constant mercaptoethanol concentration, indicating that the reactive ionic species under these conditions is the mercaptide ion. The anion is also the participating ionic species in the reaction of ME with *p*-nitrophenyl acetate (Jencks and Carriuolo, 1960). In several experiments, the total phosphate buffer concentration was varied by a factor of at least two, while maintaining constant pH and ionic strength, without affecting k_{obsd}. This result indicates the absence of additional general acid or general base catalyzed thiolysis under these conditions. The experimental data for the reactions of the four substituted phenyl esters with mercaptoethanol are given in Table IV.

Discussion

Spectrophotometric Assay for Papain. The reaction of papain with Zgly *p*-nitrophenyl ester provides a very convenient assay for enzyme activity with some advantages over others previously described. The reaction is fast, taking 1 min to complete, spectrophotometric, and is not complicated by the necessity of working with a racemized substrate (Erlanger *et al.*, 1961). In addition, the fair solubility of this ester makes it possible to work at saturating conditions, and finally, very little enzyme (about 20 μg) is required per assay. The use of this substrate would be expected to be limited to studies with fairly pure enzymes because of the lack of specificity inherent in nitrophenyl ester assays (Martin *et al.*, 1959).

Interpretation of K_m and k_{cat}. The rates of alkaline hydrolysis of the esters employed in this study vary over an 170-fold range and, with the exception of the sterically hindered *o*-nitrophenyl ester, increase as the pK_a of the alcohol moiety decreases. These observations are in accord with earlier studies on structure–reactivity relationships in ester hydrolyses (Tommila and Hinshelwood, 1938; Bruice and Schmir, 1957; Kirsch and Jencks, 1964b). Contrasting with the direct effect of structure upon reactivity as measured by saponification rates is the lack of any correlation in k_{cat} as determined from V_{max} (Table II) where these rate constants are seen to be very nearly identical. Such a result provides strong evidence that the slow step in the over-all reaction does not involve nucleophilic displacement of the alcohol moiety from the ester. The weighted average value of k_{cat} for the Zgly esters is 2.40 sec^{-1}. This figure was obtained from the values given in Table II and the relative weights assigned by program HYPER. The scatter around this value for the individual compounds as determined from the standard errors is too great to allow the definite conclusion that the rate constants are identical for all of these esters, but the similarity is far more striking than the variation, and the conclusion that k_{cat} does not reflect the lability of the ester seems to be warranted.

Values of K_m for most good substrates of papain are about 10^{-2} M (*e.g.*, Kirsch and Katchalski, 1965), but the measured K_m for the two *p*-nitrophenyl esters so far studied, those of carbobenzoxytyrosine and Zgly, are near 10^{-5} M. This 10^3-fold reduction in the apparent K_m suggested that the high reactivity of the ester is reflected in K_m rather than V_{max} and as a consequence K_m is not purely a binding constant. This supposition is clearly brought out in Figure 6, where $1/K_m$ is plotted against k_{OH^-} on a logarithmic scale. A very good linear correlation exists between these two parameters except for the point representing the *o*-nitrophenyl ester. These results are compatible with the model derived to explain the kinetics of the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate (Gutfreund and

Sturtevant, 1956)

$$E + S \overset{K_s}{\rightleftharpoons} ES_1 \xrightarrow{k_2} \underset{\displaystyle P_1}{\underset{+}{ES_2}} \xrightarrow{k_3} E + P_2 \tag{2}$$

where ES_1 is a noncovalent enzyme–substrate complex, ES_2 an acyl enzyme, P_1 the alcohol moiety of the ester, and P_2 the carboxylic acid.

The apparent K_m and k_{cat} are given by (Gutfreund and Sturtevant (1956))

$$K_m = \frac{k_3}{k_2 + k_3} K_s \tag{3}$$

and

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \tag{4}$$

For the special case where $k_2 \gg k_3$ eq 3 and 4 reduce to

$$K_m = \frac{k_3}{k_2} K_s \tag{5}$$

and

$$k_{cat} = k_3 \tag{6}$$

The following lines of evidence support the contention that the reactions studied here fall into the special case exemplified by eq 5 and 6:

(a) Values of k_{cat} have been shown to be very nearly independent of leaving group, indicating a common rate-determining step not involving the alcohol, and (b) the observation that $1/K_m$ correlates with k_{OH^-} for the various substrates (Figure 6). The implicit assumption which is made here is that K_s, the binding constant for enzyme and substrate, is relatively independent of leaving group. Zerner *et al.* (1964) have shown this to be a tenable assumption for chymotrypsin, and Whitaker and Bender (1965) have demonstrated that this constant does not differ by more than a factor of 2 in the papain-catalyzed hydrolysis of benzoylarginine ethyl ester and benzoylargininamide. Lowe and Williams (1965c), however, have calculated from their observed data in terms of the acyl-enzyme model that K_s for *n*-butyl hippurate is 20-fold lower than that observed for hippurylamide. If K_s is indeed so sensitive to the structure of the leaving group, then it becomes difficult to understand the excellent correlation reported by Bender and Kézdy (1965) for papain between k_{OH^-} and k_{cat}/K_m ($k_{cat}/K_m = k_2/K_s$ in terms of the acyl-enzyme model), since k_{OH^-} should only reflect k_2. This question of the effect of the structure of the leaving group on the observed K_s must be resolved by direct study of the acylation reaction. The fact that the correlation seen in Figure 6 does exist certainly supports the conclusion that the structure of the leaving group is not generally of great importance as a determinant of K_s, since it is unlikely that factors favoring the formation of a noncovalent complex would depend upon reactivity to nucleophilic displacement.

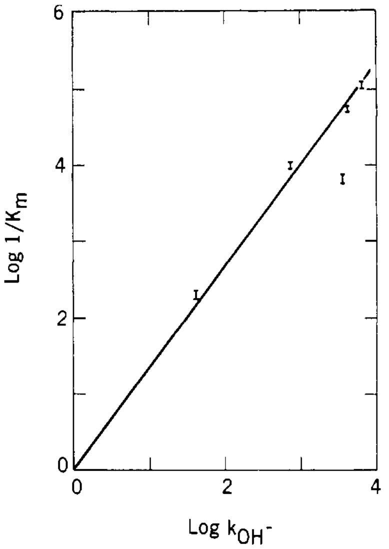
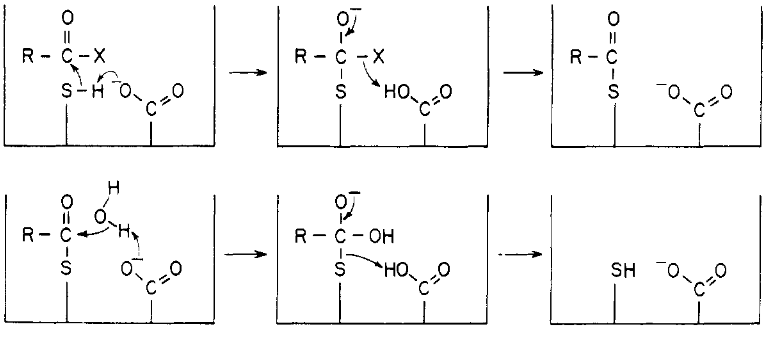


FIGURE 6: The reciprocals of the apparent Michaelis constants for the papain-catalyzed hydrolysis of esters of carbobenzoxyglycine as a function of the rates of alkaline hydrolysis at 25°. The error flags include ± the standard error as determined from program HYPER.

Further evidence that the hydrolyses of the esters studied here conform to the scheme given by eq 2 comes from the results obtained from the study of the reaction with the *o*-nitrophenyl ester where the value for K_m does not adhere to the correlation with k_{OH^-} that is observed for the less sterically hindered compounds, but k_{cat} does not differ from that observed with the other esters.[3] The deviation shown in Figure 6 for Zgly *o*-nitrophenyl ester further suggests that the steric requirements of the enzyme are much greater than those of OH^-. McDonald and Balls (1957) in-

---

[3] The constants, K_s, cannot be determined from the results reported here; however, a reasonable minimal value, assuming no involvement of the leaving group in K_s, is 0.2 M which is the measured K_m for Zgly amide determined by Sun and Tsou (1963) at 37°. By employing this value, eq 5 and 6, and the data in Table II, the following minimal values of k_2, the rate constant for acylation, can be calculated: Zgly *p*-nitrophenyl ester, 5×10^4 sec^{-1}; Zgly *m*-nitrophenyl ester, 2×10^4 sec^{-1}; Zgly phenyl ester, 4×10^3 sec^{-1}; and Zgly ethyl ester, 65 sec^{-1}. It is not clear whether the deviation of Zgly *o*-nitrophenyl ester seen in Figure 6 is due to an anomaly in K_s or in k_2, and for this reason k_2 cannot be calculated for this ester. Intramolecular reactions are frequently considered to be equivalent to intermolecular ones, with one reactant present in 10 M concentration (Bender, 1960). These values of k_2 are 10 to 50 times greater than the calculated k_{obsd} for these esters in the presence of 10 N KOH. The k_{obsd} for the *p*- and *m*-nitrophenyl esters are within a factor of 2 of the calculated k_{obsd} for the reaction with 10 M ME anion.



SCHEME I: Proposed minimal mechanism of action of papain.

vestigated the effects of positioning the nitro group at the *ortho*, *meta*, and *para* of α-*N*-benzoylarginine methyl ester. The observed K_m for the papain-catalyzed hydrolyses of these compounds decreased by a factor of 6 only when the nitro group was in the *ortho* position. The values of k_{cat} increased by only a factor of 2 when the nitro group was either in the *ortho* or in the *para* position. These observations might be interpreted in terms of a small inductive effect caused by the nitro groups as measured by k_{cat} and a larger steric effect when the nitro group is in the *ortho* position giving a more favorable K_s.

The slope of the line drawn in Figure 6 is 1.3, showing that the selectivity of the enzyme is greater than that of hydroxide ion even though its reactivity, assuming an intramolecular reaction is equivalent to a pseudo-first-order intermolecular reaction, with one of the reactants present in 10 M concentration, is much greater.[3]

The rates of reaction of the three nitrophenyl esters of Zgly with the anion of ME are about three times greater than the corresponding reactions with hydroxide ion, but the rates for the phenyl ester are about equal with the two nucleophiles, showing that the reactions of RS^- exhibit a greater sensitivity to the structure of the substrate than those of OH^-.

The Nature of the Enzyme–Substrate Intermediates. The evidence supporting the hypothesis of the existence of an acyl-enzyme intermediate in the papain-catalyzed hydrolyses of active substrates is not yet as conclusive as that amassed for chymotrypsin. The constancy of k_{cat} for esters with a common acyl, but varying alcohol moiety, as reported here and by Lowe and Williams (1965c), is consistent with such an hypothesis, but is also consistent with other possibilities such as rate-determining conformational changes. The fact that $1/K_m$ is proportional to k_{OH^-} (Figure 6), a measure of the reactivity of the substrate to nucleophilic attack, is difficult to explain by any mechanism other than that depicted by eq 2, 5, and 6. In addition, Whitaker and Bender (1965) have shown that the results of their study of pH *vs.* k_{cat} and pH *vs.* K_m for benzoylarginine ethyl ester and benzoylargininamide are accommodated by the acyl-enzyme mechanism.[4] Furthermore, the nonequilibration of the carbonyl oxygen atom of ethyl hippurate during the course of enzymatic hydrolysis excludes intermediates similar to those formed in nonenzymatic reactions (Kirsch and Katchalski, 1965). In addition to the support for this mechanism gleaned from kinetic evidence, Lowe and Williams (1964, 1965a) and Bender and Brubacher (1964) have obtained direct spectrophotometric evidence that intermediates formed during the hydrolysis of methyl thionohippurate and *trans*-cinnamoylimidazole, respectively, are the corresponding thiol esters.

It is tempting to extrapolate these interpretations to the conclusion that all papain-catalyzed reactions will follow the acyl-enzyme pathway; however, recent experiments by Caplow and Jencks (1964) on the chymotrypsin-catalyzed synthesis of acetyltyrosinehydroxamic acid and by Bernhard and Gutfreund (1965) and Barman and Gutfreund (1965) on the trypsin-catalyzed hydrolysis of benzoylarginine ethyl ester gave results that are not consistent with the simple acyl-enzyme mechanism as given by eq 2. Thus, the question of the universality of this pathway for the much less studied enzyme, papain, remains an object of further experiment, but the evidence so far accumulated in favor of an acyl-enzyme mechanism for active substrates seems sufficiently strong to warrant consideration of a possible detailed mechanism. Lowe and Williams (1965b,c) have suggested that k_{cat} represents a rate-determining nucleophilic attack of an imidazole group of the enzyme on the thiol ester produced from the original substrate combining with the active sulfhydryl group of the enzyme. They reached this conclusion principally from model studies of the hydrolysis of thiol esters aided by intramolecular nucleophilic attack of carboxylate ion. However, both the measured rate of deacylation of *trans*-cinnamoylpapain (Bender and Brubacher, 1964) and the k_{cat} for benzoylarginine ethyl ester, which is probably largely a measure of the rate of deacylation of benzoylarginylpapain (Whitaker and Bender, 1965), are slower by factors of 2.45 and 3.35, respectively, in deuterium oxide. Solvent deuterium isotope effects with k_H/k_D greater than 2 generally involve rate-determining proton transfer (Jencks and Carriuolo, 1961), whereas unassisted nucleophilic reactions of imidazole on esters give k_H/k_D approximately equal to 1.0 (Ander-

---

[4] These authors have shown, in terms of the model under consideration, that k_2 is only about threefold greater than k_3 for benzoylarginine ethyl ester. The calculations made in footnote 3 indicate $k_2 \approx 30k_3$ for the ethyl ester of Zgly.

son *et al.*, 1961). These observations would seem to indicate that the function of the reactive group under consideration would be one of proton transfer rather than of a nucleophile. The question of the nature of such a group remains an open one, although it does seem reasonable to assign the observed pK_a of 4.69 for the deacylation of *trans*-cinnamoylpapain (Bender and Brubacher, 1964) to a carboxyl group.

The mechanism shown in Scheme I is proposed as a minimal one consistent with the data so far accumulated for the papain-catalyzed hydrolysis of esters. The function of the carboxyl group in this proposal is one of proton transfer, initially by acting as a general base to increase the nucleophilicity of the un-ionized mercaptan, thus aiding the formation of a tetrahedral addition intermediate, and then, through general acid catalysis, facilitating the expulsion of the leaving group to form the thiol ester. Deacylation is postulated as the microscopic reversal of the acylation process, with water or other acyl acceptor being substituted for the sulfhydryl group of the enzyme. This mechanism assigns the alkaline pK_a of acylation to the sulfhydryl group and predicts that it would not be present in deacylation in accordance with observed results (Sluyterman, 1964; Whitaker and Bender, 1965; Kirsch and Katchalski, 1965). The high temperature sensitivity of the alkaline pK_a in a plot of k_{cat}/K_m *vs.* pH appears to correlate with the high heat of ionization of mercaptans as was first pointed out by Stockell and Smith (1957). The scheme proposed here follows the suggestion of general base assisted nucleophilic attack made by Cunningham (1957) for chymotrypsin, except for the replacement of the nucleophilic amino acid serine by cysteine and the tentative identification of the carboxylic acid as the proton-transfer reagent. Alternative mechanisms employing the kinetically indistinguishable species RS^- and R′COOH in the acylation reaction and OH^- and R′COOH in deacylation are also consistent with the observed pH dependence of papain-catalyzed reactions. Johnson (1964) has recently been able to exclude this latter possibility in one nonenzymatic displacement at acyl carbon, the general base catalyzed ethanolysis of ethyl trifluoroacetate, from a consideration of the rates of diffusion-controlled reactions.

Acknowledgment

We wish to thank Miss Dana Kramer for technical assistance during the early part of this study.

References

Anderson, B. M., Cordes, E. H., and Jencks, W. P. (1961), *J. Biol. Chem. 236*, 455.

Barkdoll, A. E., and Ross, W. F. (1944), *J. Am. Chem. Soc. 66*, 951.

Barman, T. E., and Gutfreund, H. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1243.

Bender, M. L. (1960), *Chem. Rev. 60*, 53.

Bender, M. L., and Brubacher, L. J. (1964), *J. Am. Chem. Soc. 86*, 5333.

Bender, M. L., and Kézdy, F. J. (1965), *Ann. Rev. Biochem. 34*, 49.

Bernhard, S. A., and Gutfreund, H. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1238.

Bodanszky, M., and du Vigneaud, V. (1962), *Biochem. Prepn. 9*, 110.

Bruice, T. C., and Schmir, G. L. (1957), *J. Am. Chem. Soc. 79*, 1663.

Caplow, M., and Jencks, W. P. (1964), *J. Biol. Chem. 239*, 1640.

Cleland, W. W. (1963), *Nature 198*, 463.

Cunningham, L. C. (1957) *Science 125*, 1145.

Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961), *Arch. Biochem. Biophys. 95*, 271.

Gutfreund, H., and Sturtevant, J. M. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 719.

Iselin, B., Rittel, W., Sieber, P., and Schwyzer, R. (1957), *Helv. Chim. Acta 40*, 373.

Jencks, W. P., and Carriuolo, J. (1960), *J. Am. Chem. Soc. 82*, 1778.

Jencks, W. P., and Carriuolo, J. (1961), *J. Am. Chem. Soc. 83*, 1743.

Johnson, S. L. (1964), *J. Am. Chem. Soc. 86*, 3819.

Karrer, P., and Heynemann, H. (1948), *Helv. Chim. Acta 31*, 398.

Kirsch, J. F., and Jencks, W. P. (1964a), *J. Am. Chem. Soc. 86*, 833.

Kirsch, J. F., and Jencks, W. P. (1964b), *J. Am. Chem. Soc. 86*, 837.

Kirsch, J. F., and Katchalski, E. (1965), *Biochemistry 4*, 884.

Lowe, G., and Williams, A. (1964), *Proc. Chem. Soc.*, 140.

Lowe, G., and Williams, A. (1965a), *Biochem. J. 96*, 189.

Lowe, G., and Williams, A. (1965b), *Biochem. J. 96*, 194.

Lowe, G., and Williams, A. (1965c), *Biochem. J. 96*, 199.

Martin, C. J., Golubow, J., and Axelrod, A. E. (1959), *J. Biol. Chem. 234*, 1718.

McDonald, C. E., and Balls, A. K. (1957), *J. Biol. Chem. 229*, 69.

Sluyterman, L. A. AE. (1964), *Biochim. Biophys. Acta 85*, 305.

Smith, E. L. (1958), *J. Biol. Chem. 233*, 1392.

Smith, E. L., and Parker, M. J. (1958), *J. Biol. Chem. 233*, 1387.

Stockell, A., and Smith, E. L. (1957), *J. Biol. Chem. 227*, 1.

Sun, Y.-K., and Tsou, C.-L. (1963), *Sci. Sinica* (*Peking*), *12*, 201.

Tommila, E., and Hinshelwood, C. N. (1938), *J. Chem. Soc.*, 1801.

Whitaker, J. R., and Bender, M. L. (1965), *J. Am. Chem. Soc. 87*, 2728.

Wilkinson, G. N. (1961), *Biochem. J. 80*, 324.

Zerner, B., Bond, R. P. M., and Bender, M. L. (1964), *J. Am. Chem. Soc. 86*, 3674.