The Kinetics of Papain- and Ficin-Catalysed Hydrolyses in the Presence of Alcohols

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1. The maximum rate of production of *p*-nitrophenol ($V_{\max}$) for both papain- and ficin-catalysed hydrolyses of *p*-nitrophenyl hippurate is independent of methanol concentration up to 2M for papain and 1.5M for ficin. 2. The observed catalytic constant (k_0) for the production of hippuric acid for both papain- and ficin-catalysed hydrolyses of methyl hippurate decreases with increasing methanol concentration, $1/k_0$ being linearly dependent on the methanol concentration. The $k_{\text{MeOH}}/k_{\text{H}_2\text{O}}$ ratio is determined. 3. These results provide strong evidence against general base catalysis for the rate-determining step in the deacylation of hippuryl-papain and hippuryl-ficin and probably for other specific acyl-papains and acyl-ficins. 4. The rate-determining step for the deacylation of the non-specific *trans*-cinnamoyl-papain appears to be different from that for the specific hippuryl-papain, and is probably subject to general base catalysis. It is possible, however, to accommodate all these observations in a single four-step reaction pathway. 5. Propan-2-ol did not influence the rate of production of hippuric acid for the papain-catalysed hydrolysis of methyl hippurate. A similar result has previously been reported for the ficin-catalysed hydrolysis of methyl hippurate. Ethanol and of course methanol (see 2) decrease the rate of production of hippuric acid for both papain- and ficin-catalysed hydrolyses of methyl hippurate. It is suggested that the secondary alcohol is incapable for structural reasons of approaching the bond to be hydrolysed.

The available kinetic data for the papain-catalysed hydrolyses of *N*- α -acyl-L-amino acid esters and amides obey the Michaelis-Menten equation and can be interpreted in terms of a three-step mechanism as shown in eqn. (1) (e.g. see Lowe & Williams, 1965c; Whitaker & Bender, 1965). The first intermediate (ES) is the Michaelis-Menten complex, and the second intermediate (ES') has usually been assumed to be an acyl-enzyme, in order to account for the similar catalytic constants found for alkyl and aryl esters of the same *N*- α -acyl-L-amino acid (Lowe & Williams, 1965c; Kirsch & Igelström, 1966). An alternative interpretation that has occasionally been mooted is that the ES' intermediate is not a common intermediate (e.g. it might be a tetrahedral intermediate) and that the third step (k_{+3}) is a conformational change leading to a further intermediate, which is broken down rapidly to give products P_1 and P_2 and regenerate the enzyme. No evidence in favour of this second interpretation has so far been reported for papain-catalysed hydrolyses, whereas the following observations support the proposal that ES' is an acyl-enzyme and are not compatible with the alternative suggestion. First, the acyl-

enzyme intermediate can be observed spectroscopically with methyl thionohippurate, and its steady-state concentration and subsequent decay are predictable from the Michaelis-Menten parameters obtained by pH-stat measurement (Lowe & Williams, 1964, 1965a). Secondly, Bender & Brubacher (1966) have found that papain can be 'titrated' with *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester at pH 3.2, the initial 'burst' of *p*-nitrophenol liberated being proportional to the active enzyme concentration. In addition, Brubacher (1966) has found a similar initial 'burst' of *p*-nitrophenol for the papain-catalysed hydrolysis of several *N*- α -acyl-amino acid *p*-nitrophenyl esters. It follows that with these substrates, and probably with other substrates also, the three-step mechanism involves binding (K_s) to form an enzyme-substrate complex (ES), acylation (k_{+2}) to form an acyl-enzyme (ES') plus alcohol, amine etc. (P_1) and deacylation (k_{+3}) of the acyl-enzyme to form the acid (P_2) and regenerate the enzyme:

From inhibition studies it is known that papain depends for its activity on a thiol group, and indeed recent structural studies have identified cysteine-25 as the active-centre cysteine residue (Light, Frater, Kimmel & Smith, 1964; Husain & Lowe, 1965). Direct spectroscopic observation of the acyl-enzyme generated from the methyl thionhippurate (Lowe & Williams, 1964, 1965a) and cinnamoyl-imidazole (Bender & Brubacher, 1964) provide strong evidence for a thiol group as the site of acylation. In the present paper the reasonable assumption is made that the papain-catalysed hydrolysis of hippuric esters involves an acyl-thiol-enzyme (ES').

The rate-controlling step for the deacylation of the acyl-thiol-enzyme (ES') (k_{+3} in eqn. 1) has been the subject of several tentative mechanistic proposals. (i) Smith (1958) proposed that deacylation of the acyl-thiol-enzyme was subject to nucleophilic catalysis by a carboxylate ion. (ii) Kirsch & Igelström (1966) proposed that deacylation of the acyl-thiol-enzyme was subject to general base catalysis by a carboxylate ion. (iii) Lowe & Williams (1965b) tentatively proposed that deacylation of the acyl-thiol-enzyme was subject to nucleophilic catalysis by an imidazole residue. (iv) We now add a further possible mechanism, namely that the deacylation of specific acyl-papains could be rate-controlled by a conformational change from the acyl-thiol-enzyme (ES') to another conformation of the acyl-thiol-enzyme (ES''), followed by a rapid deacylation step.

The first suggested mechanism, namely the rate-controlled nucleophilic catalysis by a carboxylate ion, was discussed by Lowe & Williams (1965b) and found to be extremely unlikely on the basis of model studies with *S*-hippurylthioglycolic acid. Subsequent work (A. W. Lake & G. Lowe, unpublished work) has shown that the rate of hydrolysis of this model compound at pH 6.34 in aqueous dioxan [in the following proportions (v/v), with dielectric constants (Landolt-Börnstein, 1959) in parentheses: 4:1 (59); 3:2 (42); 2:3 (26); 1:4 (11)] is virtually the same as in water alone. It would be unreasonable to argue therefore that a low dielectric effect on the enzyme surface could account for the different rate of hydrolysis (approx. 10⁵-fold) found between the model and the enzyme.

The second mechanistic proposal that deacylation of the acyl-enzyme (ES') is subject to general base catalysis by a carboxylate ion is made principally on the basis of the deuterium oxide kinetic isotope effect and the pH-rate profile for the rate-controlling step in the deacylation of *N*- α -benzoyl-L-argininyl-papain and *trans*-cinnamoyl-papain. The former is dependent on a group in its basic form with apparent pK_a 3.91, and k_{H_2O}/k_{D_2O} is 2.75 in the pH-independent region (Whitaker & Bender,

1965). The latter is dependent on a group in its basic form with apparent pK_a 4.69, and k_{H_2O}/k_{D_2O} is 3.35 in the pH-independent region (Bender & Brubacher, 1964). It is known, however, that the rate-controlling step in the deacylation of hippuryl-papain is independent of pH down to 3.8 (Sluyterman, 1964; Williams, 1964). It seems likely therefore that the pH- k_{+3} profile for the deacylation of *N*- α -benzoyl-L-argininyl-papain is associated with a kinetic specificity (i.e. the positively charged guanidino group of the substrate is bound to a negatively charged group, presumably a carboxylate anion, on the enzyme surface, and thereby modifying the conformation of the acyl-enzyme and enhancing the rate of the rate-controlling step for deacylation). The deacylation of *trans*-cinnamoyl-papain, unlike hippuryl-papain, also shows a pH-dependence on the basic form of a group with pK_a 4.69. The rate of deacylation, however, is about 10⁴ times lower than for *N*- α -benzoyl-L-argininyl-papain. It seems likely therefore that the deacylation of *trans*-cinnamoyl-papain involves a different rate-determining step.

The third mechanism, namely nucleophilic catalysis by an imidazole group, was tentatively suggested to account for the rate and Arrhenius parameters for the deacylation of specific acyl-papains. To account for the observed pH-independence of the deacylation of hippuryl-papain, the suggestion was made that the imidazole group was 'buried', probably by the acyl residue.

The fourth mechanism, which has not hitherto been proposed for papain-catalysed hydrolyses, is that the rate-controlling step for the deacylation of specific substrates is a conformational change from the acyl-thiol-enzyme (ES') to a conformer (ES'') followed by a more rapid breakdown to product (P₂) and enzyme.

Since only in the second mechanism is a water molecule involved directly in the rate-controlling step of deacylation, it is arguable that the observed deuterium oxide kinetic isotope effect favours this mechanism. Indeed it is precisely this argument that led Kirsch & Igelström (1966) to propose this mechanism. Whitaker & Bender (1965) also regarded this as evidence for proton transfer occurring in the rate-determining deacylation step. Now although the deuterium oxide kinetic isotope effect is a valuable method for distinguishing between general base catalysis and other mechanisms not involving water molecules, this argument is only valid if secondary isotope effects, which can arise in a variety of ways, can be estimated or excluded. With an enzyme this is patently difficult, for although it must be expected that some proton-deuteron exchange takes place it is not possible to estimate the influence of this exchange. An alternative method was sought therefore that would

provide unequivocal evidence for or against the direct participation of a water molecule in the rate-controlling deacylation step. If papain is allowed to catalyse the partial hydrolysis of methyl hippurate in a medium containing [^{14}C]methanol, radioactivity is incorporated into the methyl hippurate (Lowe & Williams, 1965c). This qualitative experiment demonstrated that methanol will compete with water in the hydrolytic step, and thus provided a means of distinguishing between general base catalysis and other mechanisms.

If general-base-catalysed deacylation of ES' occurs, eqn. (1) should be expanded to eqn. (2) when methanol is present:

where $k'_{+3} = k_{+3}[\text{H}_2\text{O}]$.

If P_1 production is followed kinetically:

$$k_0 = \frac{k_{+2}(k'_{+3} + k_{+4}[\text{MeOH}])}{k_{+2} + k'_{+3} + k_{+4}[\text{MeOH}]} \quad (3)$$

For papain-catalysed hydrolysis of *p*-nitrophenyl hippurate $k_{+2} \gg k_{+3} + k_{+4}[\text{MeOH}]$ and therefore:

$$k_0 = k'_{+3} + k_{+4}[\text{MeOH}] \quad (4)$$

If P_2 production is followed kinetically:

$$k_0 = \frac{k_{+2}k'_{+3}}{k_{+2} + k'_{+3} + k_{+4}[\text{MeOH}]} \quad (5)$$

For papain-catalysed hydrolysis of methyl hippurate $k_{+2} > k_{+3} + k_{+4}[\text{MeOH}]$ for small methanol concentrations relative to water (from the ratio of the rate constants for methanolysis and hydrolysis found in this investigation, it was possible to calculate and so not exceed the maximum methanol concentration for this condition to be valid), and therefore:

$$k_0 = k'_{+3} \quad (6)$$

If the rate-controlling step (k_{+3}) in the deacylation of ES' involves nucleophilic catalysis, a conformational change or any other conceivable process not directly involving water, eqn. (7) would apply when methanol is present:

where $k'_{+4} = k_{+4}[\text{H}_2\text{O}]$.

If P_1 production is followed kinetically:

$$k_0 = \frac{k_{+2}k_{+3}(k'_{+4} + k_{+5}[\text{MeOH}])}{k_{+2}k_{+3} + (k_{+2} + k_{+3})(k'_{+4} + k_{+5}[\text{MeOH}])} \quad (8)$$

For papain-catalysed hydrolysis of *p*-nitrophenyl hippurate $k_{+2} \gg k_{+3}$ and, if $k_{+3} \ll k'_{+4} + k_{+5}[\text{MeOH}]$, then:

$$k_0 = k_{+3} \quad (9)$$

If P_2 production is followed kinetically:

$$k_0 = \frac{k_{+2}k_{+3}k'_{+4}}{k_{+2}k_{+3} + (k_{+2} + k_{+3})(k'_{+4} + k_{+5}[\text{MeOH}])} \quad (10)$$

and if $k_{+3} \ll k'_{+4} + k_{+5}[\text{MeOH}]$ then:

$$k_0 = \frac{k_{+2}k_{+3}k'_{+4}}{(k_{+2} + k_{+3})(k'_{+4} + k_{+5}[\text{MeOH}])} \quad (11)$$

$$\frac{1}{k_0} = \frac{k_{+2} + k_{+3}}{k_{+2}k_{+3}} + \frac{(k_{+2} + k_{+3})(k_{+5}[\text{MeOH}])}{k_{+2}k_{+3}k'_{+4}} \quad (12)$$

The possibility that there is a water (or methanol) molecule bound specifically to the acyl-enzyme (ES') that is responsible for hydrolysis (or methanolysis) need not be introduced into the kinetic expression, since there is no evidence of saturation by methanol (see the Results section). It should not be concluded, however, that there is no specific binding site for water (or methanol), since, if the methanol concentration is small compared with the binding (dissociation) constant for the acyl-enzyme-methanol complex, the kinetics will be indistinguishable from the situation in which no specific binding of water or methanol takes place.

These equations are derived for steady-state conditions and it can be seen from eqns. (4) and (6) that, if general base catalysis is operating, the rate of production of P_1 will be dependent on the methanol concentration and the rate of production of P_2 will be independent of methanol concentration. If, however, an alternative mechanism is operating that can be represented by eqn. (7), it is seen from eqns. (9) and (11 or 12) that the reverse

will be observed, namely the rate of production of P_1 will be independent of the methanol concentration whereas the rate of production of P_2 will be dependent on the methanol concentration. An investigation of the influence of methanol concentration on the production of *p*-nitrophenol for the papain-catalysed hydrolysis of *p*-nitrophenyl hippurate, and on the production of hippuric acid from the papain-catalysed hydrolysis of methyl hippurate, should therefore provide decisive kinetic evidence for or against general base catalysis for the deacylation mechanism.

Although the proteolytic enzyme ficin has been much less extensively studied than papain, the available experimental evidence suggests that the mechanisms of action of the two enzymes have several important features in common. The kinetic data for the ficin-catalysed hydrolysis of *N*- α -acyl-L-amino acid esters and amides can be interpreted in terms of eqn. (1) and the pH- $V_{\max}$ (k_0) profiles for the hydrolysis of *N*- α -benzoyl-L-arginine ethyl ester (Bernhard & Gutfreund, 1956) and for methyl hippurate (this paper) are very similar to those for papain. From inhibition studies, ficin is known to depend on a thiol group, and the amino acid sequence around the active-centre cysteine residue (Wong & Liener, 1964) is remarkably similar to that found around the active-centre cysteine-25 in papain. Direct spectroscopic observation of the acyl-enzyme generated from the specific substrate methyl thionhippurate, whose steady-state concentration can be predicted from the Michaelis-Menten parameters determined by pH-stat measurements, provided strong evidence that an acyl-enzyme is formed through a thiol group (Lowe & Williams, 1964, 1965a). In addition, ficin with *p*-nitrophenyl hippurate at pH 7 gives a more rapid liberation of *p*-nitrophenol for the first turnover of the enzyme than during the steady-state hydrolysis (M. J. Hollaway & H. Gutfreund, personal communication). It follows therefore that with these substrates, and probably with other substrates also, a three-step mechanism (see eqn. 1), involving binding to form an enzyme-substrate complex (ES), acylation to form an acyl-thiol-enzyme (ES') plus alcohol, amine etc. (P_1) and deacylation to give the acid (P_2) and regenerated enzyme, outlines the rate-controlling steps. More detailed consideration is now given to the rate-controlling step in the deacylation of acyl-papains and acyl-ficins.

MATERIALS AND METHODS

Papain. Twice-recrystallized papain (British Drug Houses Ltd., Poole, Dorset) was activated with *p*-thiocresol by the method of Soejima & Shimura (1961). The concentration of *p*-thiocresol in the final preparation was

estimated to be less than $10\mu\text{M}$. The enzyme concentration was estimated by using methyl hippurate as substrate and the data of Lowe & Williams (1965c).

Ficin. Twice-recrystallized ficin (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was activated as above. The enzyme concentration was estimated by using methyl hippurate and the data of Hammond & Gutfreund (1959).

Methyl hippurate. This was prepared as described by Hammond & Gutfreund (1959) and had m.p. 80–81.5°.

***p*-Nitrophenyl hippurate.** This was prepared as described by Lowe & Williams (1965c) and had m.p. 167–168°.

***N*-Acetylimidazole.** This was prepared as described by Otting (1956) and had m.p. 103–104° (Found: C, 54.6; H, 5.5; N, 25.4; Calc. for $\text{C}_5\text{H}_8\text{N}_2\text{O}$: C, 54.45; H, 5.45; N, 25.45%), $\nu_{\max}$ 1740 cm^{-1} .

1-Acetyl-3-methylimidazolium chloride. This was prepared from *N*-methylimidazole (Häring, 1959) as described by Wolfenden & Jencks (1961) and had m.p. 107–109°.

Deuterium oxide. This was obtained from Norsk Hydro-litrisk Kvaelfroctafacieselskab, Norway.

Kinetics. (a) Titrimetric method. The Michaelis-Menten parameters for papain- and ficin-catalysed hydrolyses of methyl hippurate were determined by pH-stat as described by Lowe & Williams (1965c). By determining the initial velocities (less than 5% of total substrate had reacted) at different substrate concentrations the observed Michaelis-Menten parameters k_0 and K_m were obtained from the equation:

$$v = k_0[E][S_0]/(K_m + [S_0])$$

by using a Lineweaver-Burk plot and the method of least squares (an Autocode computer programme employing an English Electric KDF 9 digital computer enabled the least-squares results to be calculated automatically from the kinetic data).

The hydrolysis of *N*-acetylimidazole and 1-acetyl-3-methylimidazolium chloride were also followed by pH-stat. These reactions were followed to completion and the rate constants determined by the method of Guggenheim (1926).

(b) Spectrophotometric method. The papain- and ficin-catalysed hydrolyses of *p*-nitrophenyl hippurate were studied as described by Lowe & Williams (1965c).

For all the enzyme kinetics pH 6.0 was selected except where otherwise stated; k_0 is independent of pH in this region. Hydrogen ion activities and pH-meter readings in methanol-water solutions up to 68% methanol are known to be essentially identical (Bates, Paabo & Robinson, 1963). It was not therefore necessary to make any pH corrections for the competitive methanolysis-hydrolysis experiments with the enzymes, *N*-acetylimidazole or 1-acetyl-3-methylimidazolium chloride.

RESULTS

Effect of methanol on papain- and ficin-catalysed hydrolyses of *p*-nitrophenyl hippurate. The initial rate of *p*-nitrophenol production during the papain- and ficin-catalysed hydrolyses of *p*-nitrophenyl hippurate was followed spectrophotometrically at different methanol concentrations and the results are shown in Tables 1 and 2. The kinetics were performed under $V_{\max}$ conditions, so that initial

velocities represent the deacylation step. For the papain-catalysed hydrolysis the rate of *p*-nitrophenol production was unchanged up to 2.02M-methanol, but at 2.28M-methanol the rate de-

creased. In the absence of added methanol, under the same conditions, k_0 is 2.31sec.⁻¹ and K_m is 0.014mm (Lowe & Williams, 1965c). For the ficin-catalysed hydrolysis the rate of *p*-nitrophenol production was unchanged up to 1.53M-methanol, but at 1.78M-methanol the rate decreased. In the absence of added methanol, k_0 is 4.9sec.⁻¹ and K_m is 0.107mm.

Table 1. *Influence of methanol concentration on the papain-catalysed hydrolysis of p-nitrophenyl hippurate, p-nitrophenol (P₁) production being followed under V_{max} conditions*

The hydrolyses were followed spectrophotometrically at 3170Å, at 35° and pH6.0 in 0.3M-NaCl. The papain concentration was 0.1μM and the initial substrate concentration was 2mm. Each value is the average of three determinations.

Concn. of methanol (M)	k_0 (sec. ⁻¹)
0.79	2.30 ± 0.05
1.03	2.35 ± 0.15
1.28	2.25 ± 0.10
1.53	2.35 ± 0.05
1.78	2.35 ± 0.05
2.02	2.30 ± 0.10
2.28	1.95 ± 0.10

Table 2. *Influence of methanol concentration on the ficin-catalysed hydrolysis of p-nitrophenyl hippurate, p-nitrophenol (P₁) production being followed under V_{max} conditions*

The hydrolyses were followed spectrophotometrically at 3170Å, at 35° and pH6.0 in 0.3M-NaCl. The ficin concentration was 0.1μM and the initial substrate concentration was 2mm. Each value is the average of four determinations.

Concn. of methanol (M)	k_0 (sec. ⁻¹)
0.79	4.8 ± 0.1
1.03	4.9 ± 0.1
1.28	5.0 ± 0.1
1.53	4.8 ± 0.15
1.78	4.0 ± 0.2

Effect of methanol on the papain- and ficin-catalysed hydrolyses of methyl hippurate. The papain- and ficin-catalysed hydrolyses of methyl hippurate were followed by pH-stat in solvent containing different concentrations of methanol. The results in Tables 3 and 4 show that k_0 is dependent on the methanol concentration and a plot of $1/k_0$ against [methanol]/[water] should give a straight line (eqn. 12). For papain this relation holds up to a methanol concentration of 1.22M, and for ficin up to a methanol concentration of 0.23M (see Fig. 1); above these concentrations the relationship departs from linearity. By dividing the slope by the intercept of the straight lines, k_{+5}/k_{+4} is 9.5 for papain and 74.6 for ficin.

Competitive methanolysis-hydrolysis of N-acetyl-imidazole and 1-acetyl-3-methylimidazolium chloride. The hydrolysis of N-acetyl-imidazole and 1-acetyl-3-methylimidazolium chloride was followed by pH-stat at different methanol concentrations. For a given methanol-water solvent mixture:

$$\ln([OH^-]_{\infty} - [OH^-]_t) \propto -t(k'_1 + k'_2)$$

where k'_1 is the bimolecular hydrolysis constant and k'_2 is the bimolecular methanolysis constant; $[OH^-]_{\infty}$ and $[OH^-]_t$ are the pH-stat readings when the reaction is complete and at any time up to complete reaction respectively. Good linear plots of t against $\ln([OH^-]_{\infty} - [OH^-]_t)$ were obtained from which the data shown in Tables 5 and 6 were obtained.

k_0 -pH and K_m -pH profiles for ficin-catalysed hydrolysis of methyl hippurate. The hydrolyses were

Table 3. *Influence of methanol concentration on the papain-catalysed hydrolysis of methyl hippurate, hippuric acid (P₂) production being followed*

The hydrolyses were followed by pH-stat at 35° and pH6.0 in 0.3M-NaCl. Five points were used for each Lineweaver-Burk plot. The papain concentration was 1μM and the substrate concentrations were 7-25mm.

Concn. of methanol (M)	k_0 (sec. ⁻¹)	K_m (mm)	k_0/K_m (M ⁻¹ sec. ⁻¹)
0.0	2.72 ± 0.19	24.8 ± 2.5	110
0.46	2.48 ± 0.14	28.8 ± 2.4	86.1
0.77	2.38 ± 0.17	31.6 ± 3.1	75.3
0.93	2.33 ± 0.17	33.8 ± 1.8	69.0
1.24	2.21 ± 0.14	35.0 ± 3.2	63.1
1.39	2.56 ± 0.30	47.2 ± 6.0	54.2
1.55	2.53 ± 0.09	50.4 ± 1.9	50.4

Table 4. *Influence of methanol concentration on the ficin-catalysed hydrolysis of methyl hippurate, hippuric acid (P_2) production being followed*

The hydrolyses were followed by pH-stat at 35° and pH 6.0 in 0.3 M-NaCl. Five points were used for each Lineweaver-Burk plot. The ficin concentration was 1 μ M and the substrate concentrations were 7–20 mM.

Concn. of methanol (M)	k_0 (sec. ⁻¹)	K_m (mM)	k_0/K_m (M ⁻¹ sec. ⁻¹)
0.0	3.50 ± 0.13	47.4 ± 2.3	73.8
0.077	3.14 ± 0.17	72.4 ± 3.0	43.4
0.15	2.98 ± 0.18	42.7 ± 3.4	69.8
0.23	2.72 ± 0.24	42.5 ± 4.2	64.0
0.31	2.45 ± 0.21	45.2 ± 3.6	54.2
0.46	4.61 ± 0.20	105.2 ± 5.2	43.8
0.62	6.76 ± 1.50	170.2 ± 70	39.7

Table 5. *Methanolysis-hydrolysis of N-acetylimidazole*

The kinetics were followed by pH-stat at pH 6.0 and 35° in 0.3 M-NaCl. The hydrolysis rate constant determined under the same conditions was $k_1 = 4.80 \times 10^{-4}$ l.mole⁻¹min.⁻¹.

Concn. of methanol (M)	0.081	0.162	0.243	0.324
$10^2 k_2$ (l.mole ⁻¹ min. ⁻¹)	7.4	6.6	7.0	9.1

Mean methanolysis rate constant: $k_2 = 7.5 \times 10^{-2}$ l.mole⁻¹min.⁻¹; $k_2/k_1 = 156$.

Table 6. *Methanolysis-hydrolysis of 1-acetyl-3-methylimidazolium chloride*

The kinetics were followed by pH-stat at pH 6.0 and 35° in 0.3 M-NaCl. The hydrolysis rate constant determined under the same conditions was $k_1 = 7.5 \times 10^{-3}$ l.mole⁻¹min.⁻¹.

Concn. of methanol (M)	0.162	0.324	0.486	0.648	0.811
k_2 (l.mole ⁻¹ min. ⁻¹)	0.58	0.45	0.46	0.40	0.44

Mean methanolysis rate constant: $k_2 = 0.47$ l.mole⁻¹min.⁻¹; $k_2/k_1 = 63$.

followed by pH-stat at 35° in 0.3 M-sodium chloride. The k_0 -pH profile was as follows, with k_0 in sec.⁻¹ in parentheses: pH 3.75 (11.1); 4.5 (3.7); 5.5 (3.1); 6.0 (3.5); 6.5 (3.0); 7.5 (3.6). The apparent increase in k_0 at pH 3.75 is of doubtful significance because of the difficulty of following the kinetics by pH-stat at this low pH (hippuric acid pK_a 3.64). The K_m -pH profile was as follows, with K_m in mM in parentheses: pH 3.75 (371); 4.5 (75.6); 5.5 (42.5); 6.0 (50.0); 6.5 (41.2); 7.5 (58.6). Since for the ficin-catalysed hydrolysis of *p*-nitrophenyl hippurate at pH 6.0 (Table 2) k_0 is 4.8 sec.⁻¹, it follows that for the ficin-catalysed hydrolysis of methyl hippurate at the same pH k_0 (3.5 sec.⁻¹) is composite, i.e. $k_{+2}k_{+3}/k_{+2} + k_{+3}$. Accepting k_{+3} to be 4.8 sec.⁻¹, then k_{+2} is 13 sec.⁻¹. If $k_{-1} \gg k_{+2}$, i.e. if $k_0/K_m = k_{+2}/K_s$, then K_s is 185 mM.

Deuterium oxide kinetic isotope effect for the papain-catalysed hydrolysis of p-nitrophenyl hippurate. The hydrolyses were followed spectrophotometrically at 3170 Å at pH and pD 6.0 at 35° in 0.3 M-sodium chloride (cf. Lowe & Williams,

1965c). The pD was determined by using a glass electrode and a correction of +0.40 (Lumry, Smith & Glantz, 1951; Zerner & Bender, 1961). The ester in ethanol (0.10 ml., approx. 2 mM) was added to the thermally equilibrated enzyme solution (3 ml., 0.1 μ M). The results were as follows:

$$k_{0(H_2O)} = 2.3 \text{ sec.}^{-1}; K_{m(H_2O)} = 0.018 \text{ mM}$$

$$k_{0(D_2O)} = 1.35 \text{ sec.}^{-1}; K_{m(D_2O)} = 0.015 \text{ mM}$$

$$k_{0(H_2O)}/k_{0(D_2O)} = 1.7$$

Effect of ethanol and propan-2-ol on papain- and ficin-catalysed hydrolyses of methyl hippurate. The kinetics were followed by pH-stat and the results are shown in Table 8.

DISCUSSION

The results shown in Tables 1 and 2 clearly show that the rate of *p*-nitrophenol (P_1) production for both the papain- and ficin-catalysed hydrolyses of *p*-nitrophenyl hippurate are independent of the

Fig. 1. Effect of methanol on the catalytic constant (k_0) for the papain- ($\blacktriangle$) and ficin- ($\bullet$) catalysed hydrolyses of methyl hippurate at pH 6.0 in 0.3M-NaCl at 35°.

methanol concentration up to 2M for papain and 1.5M for ficin. At methanol concentrations slightly above these limits the rate of *p*-nitrophenol production is lower. The observed catalytic constant (k_0) for the production of hippuric acid (P_2) for both papain- and ficin-catalysed hydrolyses of methyl hippurate decreases with increasing methanol concentration (Tables 3 and 4), $1/k_0$ being linearly dependent on the methanol concentration as required by eqn. (12) (Fig. 1). Since k_0 is known to represent the rate-determining step for deacylation (k_{+3}) in the papain-catalysed hydrolysis of methyl hippurate and since the k_{MeOH}/k_{H_2O} ratio can be determined from the ratio of the slope to the intercept of the plot in Fig. 1, it is possible to confirm by using eqns. (5) and (6) that general base catalysis is not operative, since if it were k_{+2} would be greater than $k_{+3} + k_{+4}[MeOH]$ for the methanol concentrations used and therefore k_0 should be independent of the methanol concentration. Results reported here for the ficin-catalysed hydrolysis of *p*-nitrophenyl hippurate indicate that k_0 for the ficin-catalysed hydrolysis of methyl hippurate is a composite constant of acylation (k_{+2}) and deacylation (k_{+3}), and consequently it is not possible to use these data in the same way to confirm that general base catalysis does not obtain. Nevertheless the k_{MeOH}/k_{H_2O} ratio is important in demonstrating that the error on the data in Tables 1 and 2 is small compared with the change in k_0 that would be expected from eqn. (4) if the rate-determining step were general-base-catalysed. These results taken together therefore provide strong kinetic evidence against general base catalysis for the deacylation of hippuryl-papain, hippuryl-ficin and probably for other specific acyl-papains and acyl-ficins. Moreover, these results affirm that the rate-

Table 7. Comparison of the k_{MeOH}/k_{H_2O} ratios for papain- and ficin-catalysed hydrolyses with the model compounds *N*-acetylimidazole and 1-acetyl-3-methylimidazolium chloride

Compound	k_{MeOH}/k_{H_2O}
Hippuryl-papain (ES'')	9.5
Cinnamoyl-papain	59*
Hippuryl-ficin (ES'')	74.6
<i>N</i> -Acetylimidazole	156
1-Acetyl-3-methylimidazolium chloride	63

* M. L. Bender & L. J. Brubacher (personal communication).

determining step in the deacylation of hippuryl-papain and hippuryl-ficin is not a hydrolytic step. The available experimental evidence, however, does not allow a decision to be made between nucleophilic attack of the acyl-enzyme, a conformational change from one acyl-enzyme to another, or perhaps some other mechanism as yet not defined.

The exceptionally low rate of deacylation of *trans*-cinnamoyl-papain ($k_{25^\circ} 3.68 \times 10^{-3} \text{sec}^{-1}$ in the most favourable part of the pH-rate profile) together with the large k_{H_2O}/k_{D_2O} ratio (3.35) compared with that for the deacylation of *N*- α -benzoyl-L-argininyl-papain ($k_{25^\circ} 20.2 \text{sec}^{-1}$; $k_{H_2O}/k_{D_2O} 2.75$) suggested that for this non-specific acyl residue a different mechanism might be involved, or that if the same mechanism was involved the rate-determining step for deacylation was probably different. The influence of added nucleophiles on the deacylation of *trans*-cinnamoyl-papain has been investigated by M. L. Bender & L. J. Brubacher (personal communication). Of particular relevance here is that methanol increased the observed rate of deacylation and k_{MeOH}/k_{H_2O} was found to be 59. It seems clear therefore that the rate-determining steps for the deacylation of hippuryl-papain and *trans*-cinnamoyl-papain are different. The simplest solution to this difference is to suppose that, whereas for hippuryl-papain $k_{+3} \ll k_{+4}$, for *trans*-cinnamoyl-papain $k'_{+4} \ll k_{+3}$; however, there is no evidence at this stage to exclude other possible interpretations.

It can now be reasonably assumed that the observed deuterium oxide kinetic isotope effect for the deacylation of *N*- α -benzoyl-L-argininyl-papain ($k_{H_2O}/k_{D_2O} 2.75$; Whitaker & Bender, 1965) is due to a secondary isotope effect, whereas that for *trans*-cinnamoyl-papain ($k_{H_2O}/k_{D_2O} 3.35$; Bender & Brubacher, 1964) is due to a combination of primary and secondary isotope effects. Since for the charged benzoylargininyl residue secondary isotope effects are likely to be larger than for uncharged acyl residues, the deuterium oxide kinetic isotope effect

Table 8. *Influence of ethanol and propan-2-ol on the papain- and ficin-catalysed hydrolyses of methyl hippurate*

The hydrolyses were followed by pH-stat at 35° and pH 6.0 in 0.3M-NaCl. Five points were used for each Lineweaver-Burk plot. The enzyme concentration was 1 μ M and the substrate concentrations were 7.4–22.9 mm.

Enzyme	k_0 (sec. ⁻¹)		
	No alcohol	With ethanol (0.97M)	With propan-2-ol (0.98M)
Papain	2.72 $\pm$ 0.24	2.22 $\pm$ 0.20	2.63 $\pm$ 0.13
Ficin	3.50 $\pm$ 0.14	2.55 $\pm$ 0.15	3.37 $\pm$ 0.18

for the deacylation of hippuryl-papain was determined and k_{H_2O}/k_{D_2O} found to be 1.7. This must arise from secondary isotope effects and clearly highlights the dangers of using deuterium oxide kinetic isotope effects for elucidating the mechanism of enzyme action. However, the relative deuterium oxide kinetic isotope effects for the rate-determining step for the deacylation of cinnamoyl-papain and hippuryl-papain are consistent with the mechanism derived from the competitive methanolysis-hydrolysis experiments.

The k_{+5}/k_{+4} ratio derived from eqn. (12) and the competitive methanolysis-hydrolysis data (Tables 3 and 4 and Fig. 1) for papain- and ficin-catalysed hydrolysis of methyl hippurate are shown in Table 7, together with the k_{MeOH}/k_{H_2O} ratio for the model compounds *N*-acetylimidazole (Table 5) and 1-acetyl-3-methylimidazolium chloride (Table 6) (Wolfenden & Jencks, 1961, have shown the latter to be a satisfactory non-dissociating model for the *N*-acetylimidazolium ion). Attempts to draw firm mechanistic conclusions from such ratios, however, are known to be fraught with danger (Bender, Clement, Gunter & Kezdy, 1964). However, the quite large difference in the k_{+5}/k_{+4} ratio for the hippuryl-papain and hippuryl-ficin hints at a possible difference in mechanism in the hydrolytic step for these two enzymes.

Hammond & Gutfreund (1959) found that propan-2-ol had no effect on k_0 for the ficin-catalysed hydrolysis of methyl hippurate. We have repeated this experiment and entirely agree with this result, and we have also found the same to be true for papain (Table 8). Ethanol, on the other hand, does influence k_0 for both ficin- and papain-catalysed hydrolyses of methyl hippurate followed by pH-stat (Table 8). It would seem therefore that even if there is not specific binding of the hydrolytic water molecule only water, methanol and perhaps other low-molecular-weight primary alcohols can approach the acyl-enzyme (ES'') bond to effect hydrolysis or alcoholysis, propan-2-ol being too bulky. It is therefore noteworthy that of the considerable range of hippuric esters studied as sub-

strates for papain (Lowe & Williams, 1965c) only the isopropyl (k_0 0.44sec.⁻¹) and *n*-butyl (k_0 1.29sec.⁻¹) had appreciably lower k_0 values than methyl hippurate (k_0 2.72sec.⁻¹). This lowering of k_0 means that k_{+2} (the rate of the acylation step) is lower for these esters than for the methyl and ethyl esters. Clearly this would be so if the enzyme had difficulty in accommodating the isopropyl and *n*-butyl residues on its active site. Similar observations with secondary alcohols have been found with subtilisins (Glazer, 1966) and α -chymotrypsin (McDonald & Balls, 1956).

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