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pH-Dependence and Structure–Activity Relationships in the Papain-Catalysed Hydrolysis of Anilides

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The pH-dependence of the Michaelis–Menten parameters for the papain-catalysed hydrolysis of *N*-acetyl-L-phenylalanylglycine *p*-nitroanilide was determined. The equilibrium binding constant, K_s, is independent of pH between 3.7 and 9.3, whereas the acylation constant, k_{+2}, shows bell-shaped pH-dependence with apparent pK_a values of 4.2 and 8.2. The effect of substituents in the leaving group on the acylation constant of the papain-catalysed hydrolysis of hippuryl anilides and *N*-acetyl-L-phenylalanylglycine anilides gives rise in both series to a Hammett ρ value of −1.04. This indicates that the enzyme provides electrophilic, probably general-acid, catalysis, as well as the nucleophilic or general-base catalysis previously found. A mechanism involving a tetrahedral intermediate whose formation is general-base-catalysed and whose breakdown is general-acid-catalysed seems most likely. The similarity of the Hammett ρ values appears to exclude facilitated proton transfer as a means through which the specificity of papain is expressed.

The hydrolysis of a substrate by the proteolytic enzyme, papain, has been shown to follow a reaction pathway involving a minimum of three steps:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} ES' \xrightarrow{k_{+3}} E + P_2 + P_1$$

where ES is the Michaelis complex, ES′ the acyl-enzyme formed through the thiol group of cysteine-25, and P_2 and P_1 are the acid and alcohol or amine moiety of the hydrolysed substrate (for a recent review of the evidence see Lowe, 1970).

The specificity of papain for *N*-acetyl-L-phenylalanylglycine derivatives compared with *N*-benzoyl- and *N*-acetyl-glycine derivatives has been shown to be manifest almost exclusively in the acylation step, i.e. k_{+2} (Lowe & Yuthavong, 1971). As expected, the enzyme showed greatest specificity for those derivatives with the poorest leaving groups. With *N*-acetylglycine *p*-nitroanilide separation of the Michaelis–Menten parameters was not possible since the solubility of the substrate prevented its concentration approaching K_m, but with hippuryl *p*-nitroanilide where K_m is much smaller, separation of these parameters was possible and the observed catalytic constant, $k_{cat.}$, was that of the acylation step, k_{+2}, assuming a three-step mechanism, i.e. $k_{+2} \ll k_{+3}$. With *N*-acetyl-L-phenylalanylglycine *p*-nitroanilide the acylation constant, k_{+2}, was much larger and the catalytic constant $[k_{cat.} = k_{+2}k_{+3}/(k_{+2}+k_{+3})]$ was a composite constant of k_{+2} and k_{+3}, i.e. $k_{+2} \sim k_{+3}$ from which k_{+2} could be calculated.

It was therefore decided to investigate further the anilides of hippuric acid and *N*-acetyl-L-phenylalanylglycine to establish that K_m was the true equilibrium constant K_s and did not incorporate the formation of a possible intermediate between the Michaelis complex (ES) and the acyl-enzyme (ES′). It was also decided to establish the pH-dependence of the binding and the acylation constants with an *N*-acetyl-L-phenylalanylglycine anilide, since the pH-dependence of these constants had been determined separately for only three substrates, which were structurally similar, namely *N*-benzoyl-L-arginine amide, *N*-benzoyl-L-arginine ethyl ester (Whitaker & Bender, 1965) and *N*-benzoyl-L-citrulline methyl ester (Williams & Whitaker, 1967; Cohen & Petra, 1967). The pH-independence of the binding constant and the bell-shaped pH-dependence of the acylation constants for isopropyl hippurate and isopropyl *N*-methanesulphonylglycinate was reported (Lucas & Williams, 1969) after the completion of this investigation.

The acylation step for the α-chymotrypsin-catalysed hydrolysis of aryl trimethylacetates shows a Hammett ρ value of +1.43 and $k_{cat.}/K_m$ for aryl acetates has a ρ value of +1.8, suggesting nucleophilic or general-base catalysis (Bender & Nakamura, 1962). $k_{cat.}/K_m$ for the α-chymotrypsin-catalysed hydrolysis of the more specific aryl hippurates and aryl *N*-mesyl-L-phenylalaninates has Hammett ρ values of +0.5 and +0.45 respectively (Williams, 1970). The α-chymotrypsin-catalysed

hydrolysis of the anilides of *N*-benzoyl-L-tyrosine (Sager & Parks, 1963) and *N*-acetyl-L-tyrosine (Inagami, York & Patchornik, 1965), however, have ρ values of -1.63 and -2.0 respectively for the acylation constant, suggesting that catalysis of the acylation step also involves an electrophilic component. Most probably this takes the form of acid catalysis, either by pretransition-state proton transfer (Wang & Parker, 1967; Parker & Wang, 1968; Wang, 1968) or by general-acid catalysis (Caplow, 1969). The observation of a nitrogen kinetic-isotope effect in the α-chymotrypsin-catalysed hydrolysis of *N*-acetyl-L-tryptophan amide indicates that the carbon–nitrogen bond of the amide is broken in the rate-determining step (O'Leary & Kluetz, 1970). If a tetrahedral intermediate is involved in the acylation step, as seems likely (Hess, McConn, Ku & McConkey, 1970), then the breakdown of the tetrahedral intermediate to the acyl-enzyme must be rate-determining.

Although these observations do not demand the intervention of a tetrahedral intermediate in the acylation step of α-chymotrypsin-catalysed hydrolyses, they are consistent with a mechanism in which the formation of the tetrahedral intermediate is general-base-catalysed and its breakdown is general-acid-catalysed, the formation of the tetrahedral intermediate being rate-determining for aryl esters and its breakdown rate-determining for anilides, as is found for the OH^--catalysed hydrolysis of aryl esters (Bunton & Spatcher, 1956) and anilides (Bender & Thomas, 1961).

The overall acylation process, i.e. $k_{cat.}/K_m$, for the papain-catalysed hydrolysis of aryl hippurates has a Hammett ρ value of $+1.2$ (Lowe & Williams, 1965). Assuming a three-step-reaction pathway and $k_{-1} \gg k_{+2}$ this represents k_{+2}/K_s. Although it is not expected that K_s would be very sensitive to change in the substituent in the leaving group, the ρ value for the acylation step remains uncertain. A study of the papain-catalysed hydrolysis of the anilides of hippuric acid and *N*-acetyl-L-phenylalanylglycine should allow the effects of substituents on binding to be determined and provide further insight into the acylation mechanism. A comparison of the Hammett ρ values for the acylation step in these two series was expected to reveal to what extent the specificity of the enzyme is reflected in the bond-breaking and bond-making processes.

MATERIALS AND METHODS

Papain. Twice-crystallized papain was prepared by the method of Kimmel & Smith (1954) from dried papaya latex (Wallerstein Co., New York, N.Y., U.S.A.).

Hippuryl anilide. Hippuryl hydrazide (2g) prepared by the method of Curtius (1890) was dissolved in 2M-HCl (5ml) at 0°C. A solution of $NaNO_2$ (0.8g) in water (5ml) was added dropwise to the stirred solution, the temperature being kept below 10°C. The white precipitate of hippuryl azide that formed after about 5min was filtered off quickly and suspended in ether (50ml) at 0°C. Aniline (1g) dissolved in ether (10ml) was added dropwise to the cold stirred suspension of hippuryl azide. Stirring was continued overnight, the solution being allowed to warm gradually to room temperature. The white precipitate was collected; the crude yield was 1.6g (65%). Recrystallization from acetone gave colourless platelets, which had m.p. 211–213°C [Bergman & Fraenkel-Conrat (1937) give m.p. 212.5°C] and $\lambda_{max.}$ (in ethanol) 285nm (ϵ 1700) and 232nm (ϵ 8500) (Found: C, 71.1; H, 5.6; N, 11.1. Calc. for $C_{15}H_{14}N_2O_2$: C, 70.8; H, 5.55; N, 11.0%).

Hippuryl p-*anisidide.* This was prepared by the mixed-anhydride method as described by Lowe & Yuthavong (1971).

Hippuryl p-*chloroanilide.* This was prepared by the method described for hippuryl anilide. The quantities of reagents were the same, except that *p*-chloroaniline (1.3g) was used in place of aniline; the crude yield was 1g (30%). The product was recrystallized from ethanol–acetone; it had m.p. 223–224°C [Kirschenbaum (1961) gives m.p. 222–223°C] and $\lambda_{max.}$ (in ethanol) 249nm (ϵ 19700) (Found: C, 62.4; H, 4.8; Cl, 12.1; N, 9.6. Calc. for $C_{15}H_{13}ClN_2O_2$: C, 62.2; H, 4.5; Cl, 12.3; N, 9.7%).

Hippuryl p-*hydroxyanilide.* This was prepared by the method described for hippuryl *p*-anisidide (Lowe & Yuthavong, 1971). The quantities of reagents were doubled, and *p*-aminophenol (4.4g) was used in place of *p*-anisidine; the crude yield of *hippuryl* p-*hydroxyanilide* was 5g (45%). The product was recrystallized several times from acetone–water; it had m.p. 235–237°C and $\lambda_{max.}$ (in ethanol) 249nm (ϵ 22900) (Found: C, 66.7; H, 5.3; N, 10.4. $C_{15}H_{14}N_2O_3$ requires C, 66.7; H, 5.2; N, 10.4%).

Hippuryl p-*nitroanilide.* This was prepared as described by Lowe & Yuthavong (1971).

Acetyl-L-phenylalanylglycine anilide. This was prepared by the method described for hippuryl *p*-anisidide (Lowe & Yuthavong, 1971). The crude yield of *acetyl-L-phenylalanylglycine anilide* was 57%. The product was recrystallized from acetone–ether–light petroleum and from acetone–water; it had m.p. 208–210°C (at 198°C the crystals softened but solidified again a few degrees higher), $[\alpha]_{365}^{20}$ +35.6° (*c* 0.3 in *NN*-dimethylformamide) and $\lambda_{max.}$ (in ethanol) 242nm (ϵ 13000) (Found: C, 64.1; H, 6.4; N, 12.1. $C_{19}H_{21}N_3O_3,H_2O$ requires C, 63.9; H, 6.5; N, 11.8%).

Acetyl-L-phenylalanylglycine p-*anisidide.* This was prepared by the method described for hippuryl *p*-anisidide (Lowe & Yuthavong, 1971). The crude yield of *acetyl-L-phenylalanylglycine* p-*anisidide* was 70%. The product was recrystallized from acetone–water and acetone–ether–light petroleum; it had m.p. 211–212.5°C, $[\alpha]_D^{20}$ +49.7° (*c* 0.3 in ethanol) and $\lambda_{max.}$ (in ethanol) 251nm (ϵ 11600) (Found: C, 65.0; H, 6.2; N, 11.4. $C_{20}H_{23}N_3O_4$ requires C, 65.0; H, 6.3; N, 11.4%).

Acetyl-L-phenylalanylglycine p-*chloroanilide.* This was prepared by the method described for hippuryl *p*-anisidide (Lowe & Yuthavong, 1971). The crude yield of *acetyl-L-phenylalanylglycine* p-*chloroanilide* was 50%. The product was recrystallized from acetone–water and

acetone–ether–light petroleum; it had m.p. 234–235°C, $[\alpha]_D^{20}$ +11.0° (*c* 0.3 in *NN*-dimethylformamide) and $\lambda_{max.}$ (in ethanol) 249nm (ϵ 17100) (Found: C, 60.85; H, 5.4; Cl, 9.5; N, 11.1. $C_{19}H_{20}ClN_3O_3$ requires C, 61.0; H, 5.4; Cl, 9.5; N, 11.3%).

Acetyl-L-phenylalanylglycine m-*nitroanilide*. This was prepared by the method described for hippuryl *p*-anisidide (Lowe & Yuthavong, 1971). The crude yield of *acetyl-L-phenylalanylglycine* m-*nitroanilide* was 67%. The product was recrystallized from acetone–water and acetone–ether–light petroleum; it had m.p. 168–170°C, $[\alpha]_D^{20}$ +16.7° (*c* 0.3 in *NN*-dimethylformamide) and $\lambda_{max.}$ (in ethanol) 241nm (ϵ 21800) with a shoulder at 272nm (Found: C, 59.3; H, 5.3; N, 14.4. $C_{19}H_{20}N_4O_5$ requires C, 59.3; H, 5.25; N, 14.6%).

Acetyl-L-phenylalanylglycine p-*nitroanilide*. This was prepared by the method described by Lowe & Yuthavong (1971).

N-(*Acetyl-L-phenylalanylglycyl*)*sulphanilamide*. This was prepared by the method described for hippuryl *p*-anisidide (Lowe & Yuthavong, 1971). The crude yield of N-(*acetyl-L-phenylalanylglycyl*)*sulphanilamide* was 78%. The product was recrystallized from methanol–water and acetone–methanol–water; it had m.p. 270–272°C, $[\alpha]_D^{20}$ +10.2° (*c* 0.3 in *NN*-dimethylformamide) and $\lambda_{max.}$ (in ethanol) 259nm (ϵ 18200) (Found: C, 54.4; H, 5.2; N, 13.6; S, 8.0. $C_{19}H_{22}N_4O_5S$ requires C, 54.4; H, 5.3; N, 13.4; S, 7.7%).

Kinetics. The papain-catalysed hydrolyses of the anilides were followed spectrophotometrically at pH6.0 and at 35°C. McIlvaine's 0.04M-Na_2HPO_4–citrate buffer was used in the range pH3.7–8.05 (Dawson, Elliott, Elliott & Jones, 1969) and 0.04M-sodium borate buffer in the range pH8.2–9.3. The enzyme was activated with 1mM-EDTA and 10mM-L-cysteine for the hippuryl anilides and with 0.5mM-L-cysteine for the *N*-acetyl-L-phenylalanylglycine anilides. For the hippuryl anilides the ionic strength was 0.05M and the solutions contained 20% (v/v) of *NN*-dimethylformamide. The enzyme concentration was about 0.1mM and the substrate concentration 1.0–10mM. The initial rates (less than 5% reaction) were used and between six and ten determinations made for each Lineweaver–Burk plot. For the *N*-acetyl-L-phenylalanylglycine anilides the ionic strength was 0.3M and the solutions contained 10% (v/v) of *NN*-dimethylformamide. The enzyme concentration was 0.01mM and the substrate concentration 0.1–1.0mM. Total progress curves were used except with the *p*-nitroanilide, the products being ineffective competitive inhibitors at pH6.0. At least five points were used for each Lineweaver–Burk plot. The extinction coefficients of the anilines and anilides at the wavelength used in the kinetic studies at pH6.0 and at 35°C are: aniline, 295nm (ϵ 630); anilides, 295nm (ϵ 0); *p*-anisidine, 305nm (ϵ 1570) and 315nm (ϵ 560); hippuryl *p*-anisidide, 305nm (ϵ 100); *N*-acetyl-L-phenylalanylglycine *p*-anisidide 315nm (ϵ 0); *p*-aminophenol, 310nm (ϵ 1150); hippuryl *p*-hydroxyanilide, 310nm (ϵ 30); *p*-chloroaniline, 300nm (ϵ 1050); hippuryl *p*-chloroanilide, 300nm (ϵ 0); *N*-acetyl-L-phenylalanylglycine *p*-chloroanilide, 300nm (ϵ 70); *m*-nitroaniline, 400nm (ϵ 840); *N*-acetyl-L-phenylalanylglycine *m*-nitroanilide, 400nm (ϵ 30); *p*-nitroaniline, 410nm (ϵ 8500) and 450nm (ϵ 830); hippuryl *p*-nitroanilide, 410nm (ϵ 0); *N*-acetyl-L-phenylalanylglycine *p*-nitroanilide, 450nm (ϵ 0); sulphanilamide, 300nm (ϵ 1120); *N*-acetyl-L-phenylalanylglycine sulphanilamide, 300nm (ϵ 0).

RESULTS AND DISCUSSION

The pH-dependence of the Michaelis–Menten parameters for *N*-acetyl-L-phenylalanylglycine *p*-nitroanilide is shown in Table 1. K_m is seen to be independent of pH between 3.7 and 9.3, whereas $k_{cat.}$ shows bell-shaped pH-dependence. Since the observed catalytic constant $[k_{cat.}=k_{+2}k_{+3}/(k_{+2}+k_{+3})]$ is approaching k_{+3}, which is 6.6s^{-1} (Lowe &

Table 1. pH-*dependence of the papain-catalysed hydrolysis of acetyl-L-phenylalanylglycine* p-*nitroanilide at* 35°C *in* 10% (*v/v*) NN-*dimethylformamide*

k_{+2} is calculated from the relationship $k_{cat.}=k_{+2}k_{+3}/(k_{+2}+k_{+3})$, assuming $k_{+3}=6.6s^{-1}$ (Lowe & Yuthavong, 1971). K_s is calculated from the relationship $K_s=k_{+2}/(k_{cat.}/K_m)$, i.e. assuming $k_{-1}\gg k_{+2}$.

pH	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	k_{+2} (s^{-1})	K_s (mM)
3.7	0.31±0.06	1.05±0.21	300± 30	0.33	1.10
3.77	0.33±0.04	0.88±0.10	380± 20	0.35	0.92
3.9	0.58±0.15	1.37±0.36	430± 50	0.64	1.51
4.2	0.76±0.05	1.14±0.07	660± 20	0.85	1.29
4.6	1.11±0.19	1.09±0.19	1020± 90	1.33	1.30
5.1	1.00±0.07	0.75±0.05	1330± 50	1.18	0.89
5.5	1.20±0.07	0.86±0.05	1400± 60	1.47	1.05
6.0	1.30±0.18	0.88±0.13	1500±200	1.62	1.08
7.2	1.08±0.21	0.82±0.17	1300±100	1.29	0.99
7.7	0.98±0.14	0.91±0.12	1100±100	1.15	1.05
8.05	0.92±0.12	1.00±0.12	900±100	1.07	1.19
8.2	0.69±0.06	0.87±0.07	770± 40	0.77	1.00
8.4	0.53±0.06	0.89±0.10	590± 30	0.57	0.97
8.76	0.58±0.09	1.65±0.27	350± 20	0.64	1.83
9.0	0.49±0.27	2.22±1.23	220± 30	0.53	2.41
9.3	0.21±0.14	1.21±0.09	170± 40	0.21	1.21

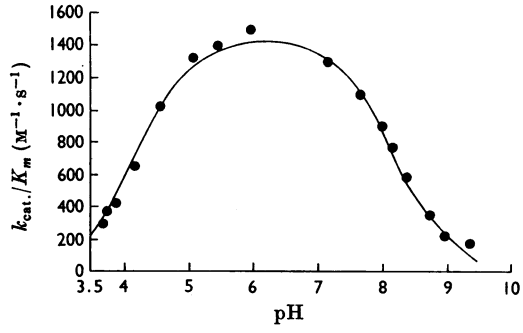

Fig. 1. pH-dependence of $k_{cat.}/K_m$ for the papain-catalysed hydrolysis of *N*-acetyl-L-phenylalanylglycine *p*-nitroanilide at 35°C in the presence of 10% (v/v) *NN*-dimethylformamide. The theoretical curve is for a limiting value of $k_{cat.}/K_m = 1450\,M^{-1}\cdot s^{-1}$ and $pK_1 = 4.2$ and $pK_2 = 8.2$.

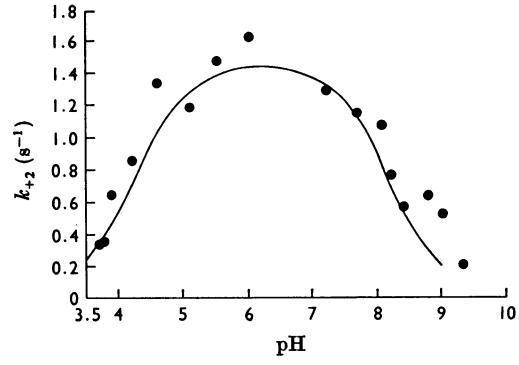

Fig. 2. pH-dependence of the acylation constant, k_2, for the papain-catalysed hydrolysis of *N*-acetyl-L-phenylalanylglycine *p*-nitroanilide at 35°C in the presence of 10% (v/v) *NN*-dimethylformamide. The theoretical curve is for a limiting value of $k_2 = 1.45\,s^{-1}$ and $pK_1 = 4.2$ and $pK_2 = 8.2$.

Yuthavong, 1971), it must be a composite constant of k_{+2} and k_{+3}, i.e. $k_{+2} \sim k_{+3}$. Since k_{+3} is known to be independent of pH between 3.7 and 7.0, and since k_{+3} is pH-independent up to pH 9.3 for four other specific substrates (Yuthavong, 1969), k_{+2} and K_s were calculated by assuming a three-step-reaction pathway in which k_{+3} is independent of pH between 3.7 and 9.3 and $k_{-1} \gg k_{+2}$. The latter assumption is reasonable since k_{+1} must be approaching the rate constant for diffusion control and K_m is about 1 mM, so that k_{-1} must be many orders of magnitude greater than k_{+2}. $k_{cat.}/K_m$ can, of course, be more accurately determined than the individual parameters, and this is reflected in the better fit of the theoretical curve for the pH-dependence of $k_{cat.}/K_m$ (Fig. 1) compared with k_{+2} (Fig. 2); K_s is pH-independent. The similarity of these pK_a values to those previously obtained from the pH-dependence of $k_{cat.}/K_m$ for other substrates (for a recent summary see Lowe, 1970) rules out the possibility that K_m incorporates the formation of a tetrahedral intermediate since k_{+2} would then represent the breakdown of the tetrahedral intermediate or a conformational change which would not be expected to show the observed bell-shaped pH-dependence. This conclusion was further supported by the absence of any detectable absorption above 350 nm immediately after mixing of a solution of papain and hippuryl *p*-nitroanilide at pH 6.0 to give final concentrations of 0.15 mM and 0.3 mM respectively. Assuming that the tetrahedral intermediate would have similar absorption properties to *p*-nitroaniline ($\lambda_{max.}$ 380 nm; ϵ 10000) if the equilibrium constant for its formation from the enzyme–substrate complex were greater than unity, an extinction of about 0.1 near 380 nm should have been observed.

The Michaelis–Menten parameters for the papain-catalysed hydrolysis of hippuryl anilides and *N*-acetyl-L-phenylalanylglycine anilides are shown in Tables 2 and 3 respectively. For the hippuryl anilides $k_{cat.}$ is at least 100-fold smaller than k_{+3} ($2.7\,s^{-1}$) and can be taken to represent k_{+2}; K_m therefore is equivalent to K_s, assuming $k_{-1} \gg k_{+2}$. For the *N*-acetyl-L-phenylalanylglycine anilides k_{+2} and K_s are calculated (see Table 3) by using $k_{+3} = 6.6\,s^{-1}$ (Lowe & Yuthavong, 1971) and assuming a three-step-reaction pathway. Although there is some indication that substituents in the leaving group modify the binding constant, the effect is small and the Hammett plot against σ scattered. The acylation constants on the other hand show a very clear trend, and the Hammett plots are shown in Figs. 3 and 4. The Hammett ρ values of −1.04 in each series show that the acylation step involves electrophilic catalysis, which is most likely to involve some degree of proton transfer in the transition state leading to the acyl-enzyme for these substrates.

The small variation of K_s with change in substituent compared with that between the change from amide ($K_s = 220$ mM for hippurylamide; Lowe & Williams, 1965) to anilide ($K_s = 19.4$ mM for hippuryl anilide) suggests that hydrophobic interactions are more significant than electronic changes in the binding contribution of the leaving group. This suggests that the Hammett ρ value of +1.2 for $k_{cat.}/K_m$ for the papain-catalysed hydrolysis of aryl hippurates (Lowe & Williams, 1965) is a reasonable approximation to the true ρ value for the acylation step, i.e. k_{+2}. The similarity of the Hammett ρ values for the acylation step in the α-chymotrypsin-catalysed hydrolysis of aryl esters (+1.43) (Bender & Nakamura, 1962) and the

Table 2. *Papain-catalysed hydrolyses of hippuryl anilides*

Hydrolyses were carried out at pH 6.0 and at 35°C in a solution containing 20% (v/v) of *NN*-dimethylformamide. The Hammett σ values are from Barlin & Perrin (1966).

Hippuric anilide	$k_{cat.}$ (ms^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	σ
p-OH	45.3	57.1	0.79±0.05	−0.37
p-O-CH_3	24.0	15.9	1.51±0.06	−0.27
p-H	15.1	19.4	0.77±0.06	0
p-Cl	6.7	6.5	1.03±0.05	+0.23
p-NO_2	2.6	7.2	0.36±0.03	+0.78

Table 3. *Papain-catalysed hydrolysis of* N-*acetyl*-L-*phenylalanylglycine anilides*

Hydrolyses were carried out at pH 6.0 and at 35°C in a solution containing 10% (v/v) of *NN*-dimethylformamide. k_{+2} was calculated from the relationship $k_{cat.}=k_{+2}k_{+3}/(k_{+2}+k_{+3})$ by using $k_{+3}=6.6\,s^{-1}$ (Lowe & Yuthavong, 1971). K_s was calculated from the relationship $K_s=k_{+2}/(k_{cat.}/K_m)$, i.e. assuming $k_{-1}\gg k_{+2}$. The Hammett σ values are from Barlin & Perrin (1966).

Anilide	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	k_{+2} (s^{-1})	K_s (mM)	σ
p-O-CH_3	5.1	0.96	5300±100	22.4	4.2	−0.27
p-H	4.3	1.55	2800±200	12.5	4.3	0
p-Cl	3.7	0.78	4800±200	8.5	1.78	+0.23
p-SO_2-NH_2	2.4	0.79	3100±300	3.9	1.26	+0.57
m-NO_2	1.6	0.78	2100±100	2.2	0.96	+0.71
p-NO_2	1.3	0.88	1500±200	1.6	1.07	+0.78

anilides of *N*-benzoyl-L-tyrosine (−1.63) and *N*-acetyl-L-tyrosine (−2.0) (Sager & Parks, 1963; Inagami *et al.* 1965) compared with those for the papain-catalysed hydrolysis of substrates with similar leaving groups suggests that the bond-breaking and bond-making processes are probably catalysed chemically by similar mechanisms, e.g. general-base–general-acid catalysis. Although it is not possible to rule out a direct displacement mechanism involving concerted general-base–general-acid catalysis, this seems less likely by analogy with acid- and base-catalysed hydrolysis of simple esters and amides where the involvement of tetrahedral intermediates has been established beyond reasonable doubt (see Jencks, 1969; O'Connor, 1970 for recent reviews). Moreover in the hydrolysis of anilides and amides evidence has been presented for the partial or complete proton transfer to the leaving group in the breakdown of the tetrahedral intermediate (Bender & Thomas, 1961; Eriksson & Bratt, 1967; Pratt & Lawlor, 1969), except that when the leaving group is a very weak base it may depart as its anion (Pollack & Bender, 1970). In model studies designed to study the breakdown of the tetrahedral intermediate of the type that would be encountered in the reaction of thiols with amides or the aminolysis of thiol esters, the breakdown of the tetrahedral intermediate to amine and thiol ester takes place through the neutral dipolar tetrahedral intermediate, i.e. the leaving group must be protonated (Chaturvedi, MacMahon & Schmir, 1967; Chaturvedi & Schmir, 1969; Barnett & Jencks, 1969). It seems probable therefore from the observed Hammett ρ values for

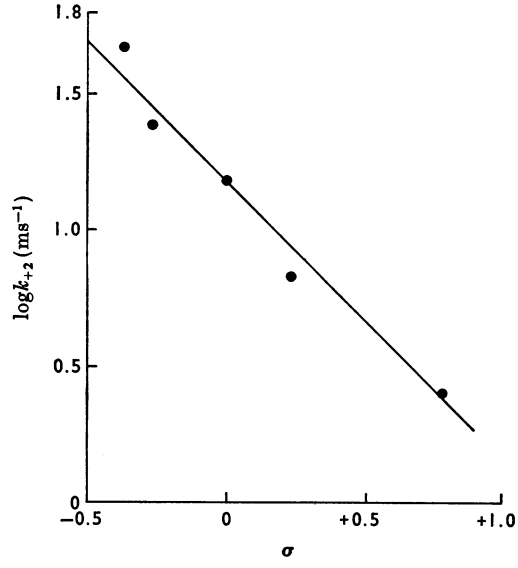

Fig. 3. Hammett plot of the acylation constant, k_{+2}, for the papain-catalysed hydrolysis of hippuryl anilides. The Hammett ρ value is −1.04 with a correlation coefficient of 0.98.

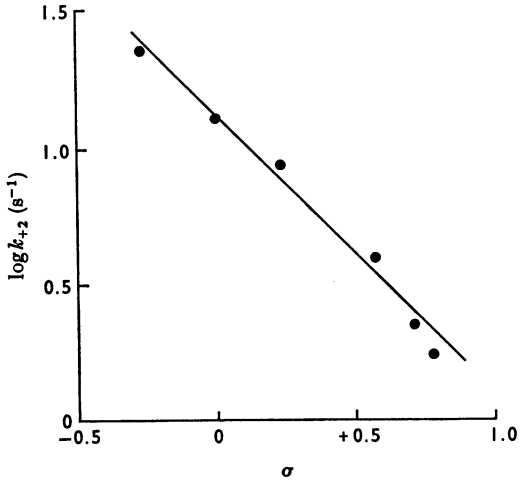

Fig. 4. Hammett plot of the acylation constant, k_{+2}, for the papain-catalysed hydrolysis of *N*-acetyl-L-phenylalanylglycine anilides. The Hammett ρ value is −1.04 with a correlation coefficient of 0.99.

the papain-catalysed hydrolysis of aryl esters and anilides that the lowest-energy reaction pathway involves a tetrahedral intermediate whose formation is general-base-catalysed and whose breakdown is general-acid-catalysed.

The similarity between the Hammett ρ values for the acylation step, k_{+2}, in the papain-catalysed hydrolysis of hippuryl anilides and *N*-acetyl-L-phenylalanylglycine anilides, in spite of the rate constants differing about 1000-fold, makes facilitated proton transfer (Wang & Parker, 1967) an unlikely explanation for the specificity of papain. These facts are, however, consistent with the view that the difference in acylation rate constants in the two series is due to differences of strain in the Michaelis complex (ES) and that this strain is essentially released in the transition state of the acylation step for these substrates. It has been suggested (Lowe & Yuthavong, 1971) that if a tetrahedral intermediate is involved in the reaction pathway it will not be strain-free. If this suggestion is correct then the similarity of the Hammett ρ values provides further support that for anilides the transition state involves the breakdown of the tetrahedral intermediate to the acyl-enzyme. Compelling proof, however, for the intervention of such an intermediate is still required.

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