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The kinetics of hydrolysis of some synthetic substrates containing neutral hydrophilic groups by pig pepsin and chicken liver cathepsin D

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1. Several peptides containing either of the sequences -Phe(NO_2)-Trp- and -Phe(NO_2)-Phe- and an uncharged hydrophilic group were synthesized, and the steady-state kinetics of their hydrolysis by pig pepsin (EC 3.4.23.1) and chicken liver cathepsin D (EC 3.4.23.5) were determined. Despite the presence of a hydrophilic group to increase substrate solubility, it was not possible to achieve the condition $[S]_0 \gg K_m$, and, in some cases, only values of $k_{cat.}/K_m$ could be determined by measuring the first-order rate constant when $[S]_0 \ll K_m$. 2. Occupancy of the P_2 and P_3 sites considerably enhanced the specificity constant, and alanine was more effective than glycine at site P_2. 3. The specificity constants for the hydrolysis by pepsin of those substrates in the present series that contain an amino acid residue at site P_3 are considerably lower than for comparable substrates containing a cationic group. This difference does not apply to cathepsin D. 4. Hydrolyses with cathepsin D commonly exhibited a lag phase, and a possible explanation for this is given.

During previous studies on the specificity of pepsin towards synthetic substrates, either dipeptides (Jackson *et al.*, 1966; Clement *et al.*, 1968; Denburg *et al.*, 1968) or larger peptides with cationic groups to enhance solubility in water (Fruton, 1976) have commonly been used. Since the rate of cleavage of substrates by pepsin is known to be markedly affected by binding at subsites several residues away from the scissile bond, dipeptides have obvious limitations for specificity studies and are usually poor substrates. The inclusion of imidazolium or pyridinium groups in the larger cationic substrates, however, may have profound effects on the catalytic process in an active-site cleft that contains two aspartic acid residues. The specificity of cathepsin D is similar to but even more limited than that of pepsin, since small peptides are cleaved only very slowly or not at all.

We have synthesized several neutral peptides containing either of the sequences -Phe(NO_2)-Trp- and -Phe(NO_2)-Phe- and determined the kinetic parameters of their cleavage by pepsin and cathepsin D by using the spectrophotometric method of Inouye & Fruton (1967). In attempts to obtain substrates with an adequate solubility in water, a *C*-terminal 2-hydroxyethylamide or a D-glucosaminol residue linked via an amide bond was incorporated into the structure of the substrates.

Abbreviations used: Z, benzyloxycarbonyl; Boc, t-butoxycarbonyl.

Experimental

Materials

Pepsin and cathepsin D were prepared as described previously (Irvine & Elmore, 1979). 2-Methoxyethanol of spectrophotometric grade was supplied by the Aldrich Chemical Co., Milwaukee, WI, U.S.A. The following compounds were synthesized as described in the literature H-Phe(NO_2)-OH,H_2O (Bergel & Stock, 1954); $^+H_2$-Phe(NO_2)-OMe,Cl^- (DeGraw *et al.*, 1968); Boc-Ala-OH (Anderson & McGregor, 1957); Boc-Phe(NO_2)-OH (Schwyzer & Caviezel, 1971); Z-Ala-OH (Marchiori *et al.*, 1963); Z-Gly-OPh(2,4,5-Cl_3) (Pless & Boissonnas, 1963); H-Trp-$NHCH_2CH_2OH$ (Sarges & Witkop, 1965); D-glucosaminol hydrochloride was synthesized by reduction of *N*-acetyl-D-glucosamine with $NaBH_4$ (Bragg & Hough, 1957) followed by hydrolysis with refluxing 2M-HCl (van den Eijnden *et al.*, 1976). Other intermediates were purchased from Sigma Chemical Co., Poole, Dorset, U.K. The remaining intermediates and all substrates were synthesized as listed in Table 1. All compounds listed were shown

Table 1. *Synthesis of substrates and intermediates*

R = CH_2–CH_2OH; R′ = $CH(CH_2OH)$–$[CHOH]_3$–CH_2OH. Methods: (*a*) mixed-anhydride method with iBu–O–COCl and *N*-methylmorpholine in tetrahydrofuran or *NN*-dimethylformamide; (*b*) 'active' ester synthesis with either *p*-nitrophenyl or 2,4,5-trichlorophenyl ester.

	Compound	Starting materials	Method (yield)	Solvent for recrystallization	M.p.
(I)	Z-Phe(NO_2)-O^- $(C_6H_{11})_2NH_2^+$	ZCl, H-Phe(NO_2)-OH	pH 7.5–8.5 in H_2O/EtOH (80%)	MeOH/Et_2O	195–197°C
(II)	Z-Gly-Gly-Phe(NO_2)-OMe	Z-Gly-Gly, H-Phe(NO_2)-OMe	(*a*) (74%)	EtOH/petrol (b.p. 40–60°C)	115–117°C
(III)	Z-Gly-Gly-Phe(NO_2)-OH	(II)	NaOH/EtOH (42%)	MeOH	162–163°C
(IV)	Z-Phe(NO_2)-Trp-NHR	(I), H-Trp-NHR	(*a*) (79%)	EtOAc	184–185°C
(V)	Boc-Phe(NO_2)-Trp-NHR	Boc-Phe(NO_2)-OH, H-Trp-NHR	(*a*) (72%)	EtOH/petrol (b.p. 40–60°C)	155°C
(VI)	H-Phe(NO_2)-Trp-NHR	(V)	HCl/Et_2O/CH_2Cl_2 (90%)	$CHCl_3$	167–169°C
(VII)	Z-GlyPhe(NO_2)-Trp-NHR	Z-Gly-ONp, (VI)	(*b*) (70%)	MeOH/CH_2Cl_2	190°C*
(VIII)	Z-Ala-Phe(NO_2)-Trp-NHR	Z-Ala-ONp, (VI)	(*b*) (78%)	MeOH	214–216°C
(IX)	Boc-Ala-Phe(NO_2)-Trp-NHR	Boc-Ala-OH, (VI)	(*a*) (80%)	EtOAc/petrol (b.p. 40–60°C)	199–201°C
(X)	Cl^- $^+H_3$-Ala-Phe(NO_2)-Trp-NHR	(IX)	HCl/Et_2O/CH_2Cl_2 (84%)	MeOH/Et_2O	230°C (decomp.)
(XI)	Z-Gly-Gly-Phe(NO_2)-Trp-NHR	Z-Gly-Gly-ONp, (VI)	(*b*) (78%)	MeOH/CH_2Cl_2	180°C*
(XII)	Z-Gly-Ala-Phe(NO_2)-Trp-NHR	Z-Gly-OH, (X)	(*a*) (71%)	EtOH/H_2O	185°C*
(XIII)	Z-Ala-Ala-Phe(NO_2)-Trp-NHR	Z-Ala-OH, (X)	(*a*) (70%)	EtOAc	243–245°C (decomp.)
(XIV)	Z-Phe-Gly-OMe	Z-Phe-OH, H-Gly-OMe	(*a*) (85%)	EtOH/H_2O	116–117°C
(XV)	Boc-Phe(NO_2)-Phe-Gly-OMe	H-Phe-Gly-OMe [from (XIV)], Boc-Phe(NO_2)-ONp	(*b*) (60%)	EtOH	174–175°C
(XVI)	Z-Gly-Gly-Phe(NO_2)-Phe-Gly-OMe	Z-Gly-Gly-OH, H-Phe(NO_2)-Phe-Gly-OMe [from (XV)]	(*a*) (75%)	iPrOH/petrol (b.p. 60–80°C)	197–200°C (decomp.)
(XVII)	Z-Gly-NHR′	Z-Gly-OPh(2,4,5-Cl_3), D-glucosaminol (R′NH_2)	(*b*) (65%)	H_2O	146–147°C (decomp.)
(XVIII)	Cl^- $^+H_3$-Gly-NHR′	(XVII)	H_2/Pd black (81%)	EtOH/H_2O	116–117°C (decomp.)
(XIX)	Z-Gly-Gly-Phe(NO_2)-Phe-Gly-Gly-NHR′	Z-Gly-Gly-Phe(NO_2)-Phe-Gly-OH [from (XVI)], (XVIII)	(*a*) (63%)	EtOH/H_2O	180–182°C (decomp.)

* These compounds did not melt sharply if heated slowly from room temperature; reasonably sharp and reproducible behaviour was observed when the melting-point apparatus was preheated to within about 20°C of the melting point reported.

to be pure by t.l.c., and all gave satisfactory microanalyses.

Kinetic and computational techniques

The operational molarity of solutions of pepsin and cathepsin D was determined by the method of Irvine & Elmore (1979). The kinetics of hydrolysis of substrates were monitored spectrophotometrically with a Cary 118C double-beam recording spectrophotometer equipped with a cell jacket maintained at 37.0 ± 0.1°C. Chart speeds of 5 μm/s (2×10^{-4} in/s) to 250 μm/s (1×10^{-2} in/s) and full-scale deflexion equivalent to an absorbance of either 0.1 or 0.05 were used. The response period on the recorder pen was either 5 s or 25 s. A tungsten lamp at high intensity was used as light-source with constant slit width of 3 mm and with automatic gain on the photomultiplier. The rate of hydrolysis was measured by monitoring the liberation of the -Phe(NO_2) carboxylate anion as described by Inouye & Fruton (1967), who found that cleavage of the Phe(NO_2)-Phe bond at pH 4 could be monitored by measuring the change in absorption at 310 nm ($\Delta\varepsilon = 800\ M^{-1} \cdot cm^{-1}$). For the single substrate containing this group that we studied, we found $\Delta\varepsilon = 737\ M^{-1} \cdot cm^{-1}$ in 50 mM-sodium acetate buffer, pH 4.0. For substrates containing the Phe(NO_2)–Trp bond, we found that $\Delta\varepsilon_{310} = 620$–$680\ M^{-1} \cdot cm^{-1}$

in 50mM-sodium acetate buffer, pH4.0, containing 1% (v/v) 2-methoxyethanol. In the case of Ala-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ we were able to use relatively high substrate concentrations, and above 0.326mM it was convenient to monitor the progress of hydrolysis at 326nm ($\Delta\varepsilon = 295\ M^{-1}\cdot cm^{-1}$).

Because of the low solubility of the substrates, the following procedure was adopted. A known weight of the substrate was dissolved in 2-methoxyethanol (1ml). The solution was kept in a boiling-water bath during the addition of hot 50mM-sodium acetate buffer, pH4.0, to give a total volume of about 5ml. This solution was added to a volumetric flask containing buffer at 80°C, and the solution was diluted to 100ml (calibrated with hot water). The solution was cooled to about 45°C, and serial dilutions were made, the making up to volume being with buffer/2-methoxyethanol (99:1, v/v). To avoid crystallization of the substrate, each solution was maintained at 45°C until immediately before use. Determinations of the initial velocity of hydrolysis were performed by adding to a solution (3.0ml) of the substrate in a 1cm cuvette, equilibrated in the sample chamber of the cell compartment, a solution (25μl) of pepsin in 50mM-sodium acetate buffer, pH5.5, or a solution (100μl) of cathepsin D in 50mM-sodium acetate buffer, pH5.1. The solution was stirred and the absorbance was recorded during 10–60min. The reference chamber of the cell compartment contained a solution (3.0ml) of the substrate to which no enzyme was added.

Experiments performed to determine the pseudo-first-order rate constants for the hydrolysis of dilute solutions of substrates were performed by adding to a solution of the substrate (25.0–28.0ml) in a 10cm cylindrical cuvette in the sample chamber of the cell compartment a solution (25–100μl) of pepsin in 50mM-sodium acetate buffer, pH5.5, or a solution (0.5–3.0ml) of cathepsin D in 50mM-sodium acetate buffer/2-methoxyethanol (99:1, v/v). The solution was mixed by inversion and the absorbance was recorded until it became constant. The reference chamber of the cell compartment contained a solution (25.0–28.0ml) of the substrate to which no enzyme was added.

Initial velocities and preliminary estimates of K_m and $k_{cat.}$ values were computed by the method of Elmore *et al.* (1963). The K_m and $k_{cat.}$ values were refined by applying the least-squares method directly to the Michaelis–Menten equation (Roberts, 1977). Preliminary values of psuedo-first-order rate constants and amplitudes were obtained from a weighted least-squares fit to the equation:

$$\ln(A_\infty - A_0)/(A_\infty - A_t) = kt$$

and then refined iteratively by applying a non-linear least-squares fit to the equation:

$$A_t = A_\infty - (A_\infty - A_0)e^{-kt}$$

Results

It was shown by t.l.c. of the products of peptic hydrolysis of Z-Gly-Gly-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ that only Z-Gly-Gly-Phe(NO_2)-OH and Trp-$NHCH_2CH_2OH$ were formed. From consideration of this result and the known specificity of pepsin and cathepsin D towards substrates similar to those used in the present study, it is clear that only the Phe(NO_2)–Trp bond is susceptible to cleavage by pepsin and cathepsin D. The kinetic parameters determined for the hydrolysis of several substrates by pepsin and cathepsin D are summarized in Tables 2 and 3. Owing to the insolubility of the neutral substrates in water, it was necessary to incorporate an organic solvent into the medium. Preliminary experiments on the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe in 40mM-sodium citrate buffer, pH4.0, containing 1% (v/v) of a number of organic solvents showed that the presence of these solvents caused a decrease in the rate of hydrolysis

Table 2. *Kinetic parameters for pepsin-catalysed reactions*

All determinations of K_m and $k_{cat.}$ were made with 11 or 12 substrate concentrations at 37°C in 50mM-sodium acetate buffer, pH4.0, containing 1% (v/v) 2-methoxyethanol. Organic solvent was omitted with substrate (XIX).

Substrate	$[S]_0$ (μM)	$[E]_T$ (nM)	$k_{cat.} \pm$ S.E.M. (s^{-1})	$K_m \pm$ S.E.M. (μM)	$k_{cat.}/K_m \pm$ S.E.M. ($M^{-1}\cdot s^{-1}$)
(X)	86.9–1090	1340	0.0730 ± 0.0037	1370 ± 90	53.1 ± 0.9
(IV)*	7.34	185	—	—	3510 ± 20
(VII)	22.7–190	45.1	4.05 ± 0.19	309 ± 19	13100 ± 200
(VIII)*	8.01	4.87	—	—	57400 ± 300
(XI)	28.8–240	35.2	8.15 ± 0.37	499 ± 29	16300 ± 200
(XII)	24.8–207	16.1	41.2 ± 1.1	266 ± 10	155000 ± 2000
(XIII)	13.4–113	10.1	25.6 ± 1.4	196 ± 14	131000 ± 2000
(XIX)*	20.4	190	—	—	16000 ± 700

* $k_{cat.}/K_m$ was determined from first-order rate constants at low $[S]_0$.

Table 3. *Kinetic parameters for cathepsin D-catalysed reactions*

Kinetic determinations were made in 50mM-sodium acetate buffer, pH4.0, containing 1% (v/v) 2-methoxyethanol at 37°C.

Substrate	$[S]_0$ (μM)	$[E]_T$ (nM)	$k_{cat.}/K_m$ + S.E.M. ($M^{-1} \cdot s^{-1}$)
(VII)	7.15	67.8	563 ± 13
(VIII)	7.99	73.6	1160 ± 10
(XI)	3.94	203	574 ± 6
(XII)	1.84	75.1	8790 ± 10
(XIII)	2.65	36.8	13300 ± 100

by pepsin. The smallest decrease was observed with methanol and was only slightly less than that caused by 2-methoxyethanol. Formamide, *NN*-dimethylformamide and *N*-methylpyrrolid-2-one respectively caused increasing degrees of inhibition. Most of the peptides used in the present study were not very soluble in methanol, but all were readily soluble in 2-methoxyethanol. With one exception noted below, all the kinetic studies were therefore performed in 50mM-sodium acetate buffer, pH4.0, containing 1% (v/v) 2-methoxyethanol. A lower concentration of organic solvent resulted in decreased substrate solubility, whereas higher concentrations were avoided to lessen the possibility of enzyme denaturation. In the above solvent most of the substrates were sufficiently soluble to allow a determination of K_m for hydrolysis by pepsin. In three cases [Z-Phe(NO_2)-Trp-$NHCH_2CH_2OH$, Z-Ala-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ and Z-Gly-Gly-Phe(NO_2)-Phe-Gly-Gly-glucosaminol], where the solubility was less than 0.1mM, however, the kinetics of hydrolysis by pepsin were monitored at substrate concentrations sufficiently low for first-order kinetics to be obeyed. From the observed first-order rate constant ($k_{cat.}[E]_T/K_m$) and the measured operational molarity of the enzyme, the specificity constant could be determined. We were able to avoid using an organic solvent with Z-Gly-Gly-Phe(NO_2)-Phe-Gly-Gly-NHR' {R' = $CH(CH_2OH)[CHOH]_3CH_2OH$} at low substrate concentrations, but the solubility was still too low to permit determination of $k_{cat.}$ and K_m separately.

Several difficulties were encountered in the kinetic experiments with cathepsin D. The rate of hydrolysis of all the substrates by cathepsin D was considerably lower than the rate of hydrolysis by pepsin. Longer runs and/or higher concentrations of enzyme were therefore required. When the concentration of the stock solution of cathepsin D was 20μM and 100μl was added to 28.0ml of the substrate solution, a pronounced lag phase was observed. This lag period, which preceded Michaelis–Menten kinetics, depended on the nature and the concentration of the substrate and was longest with low concentrations of poor substrates. The lag phase was not affected by preincubation of the enzyme solution in 1% (v/v) 2-methoxyethanol. The recorded absorbance changes did not differ when measured against air, KNO_3 solution or a solution of substrate to which no enzyme had been added. The lag phase was nearly eliminated by diluting the stock enzyme solution (1ml) with 50mM-sodium acetate buffer (pH4.0)/2-methoxyethanol (99:1, v/v) (9ml) and incubating this solution for 2h before addition to the substrate. A substrate inhibition effect was also noted with substrate concentrations of Z-Gly-Gly-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ greater than 1.7mM and Z-Ala-Ala-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ greater than 0.09mM. Because of these technical difficulties encountered in the cathepsin D-catalysed hydrolyses, kinetic measurements for all substrates were made at low substrate concentrations, and first-order rate constants were determined from which $k_{cat.}/K_m$ could be obtained. The rate of hydrolysis of Z-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ by cathepsin D was too low to be measured accurately. No hydrolysis of Ala-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ by cathepsin D was observed during 5h when the substrate concentration was 1.087mM and the enzyme concentration was about 0.6μM.

Discussion

An initial lag phase was observed during the cathepsin D-catalysed hydrolysis of all the substrates studied. The lag period, which was less prolonged with the better substrates, was considerably diminished by dilution of the enzyme stock solution 2h before its addition to the substrate solution. One explanation for this behaviour is that cathepsin D exists in an associated form in the stock solution (20μM) and slowly dissociates on dilution to give a more active form, the process being accelerated in the presence of a good substrate. Interactions between enzyme molecules might be mediated through the hydrophobic 'tail region' envisaged by Huang *et al.* (1979). Such a hydrophobic region on the surface of the enzyme would also account for the quantitative elution of cathepsin D from Sephadex G-25 by water/2-methylpropan-2-ol (49:1, v/v) but not by water (Irvine & Elmore, 1979). A less-reactive oligomeric form of cathepsin D would also account for the incomplete inactivation of the enzyme (20μM) by a large excess of *N*-diazoacetyl-L-phenylalanine 3-phenylpropylamide, whereas 5μM-enzyme was totally inactivated under the same conditions (Irvine & Elmore, 1979). An alternative explanation for the lag period is that the substrates caused differing degrees of activation

Table 4. *Effect of amino acid residues at the P_2 and P_3 sites on the kinetics of action of pepsin and cathepsin D*

Data in columns 2 and 4 relate to the hydrolysis of X-Phe-Phe-O-[CH_2]$_3$-  at pH3.5 at 37°C and are taken from Sampath-Kumar & Fruton (1974). Data in columns 3 and 5 relate to the hydrolysis of X-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ in 50mM-sodium acetate buffer containing 1% 2-methoxyethanol at pH4.0 at 37°C and are taken from Tables 2 and 3. The data in columns 2–5 are the values of $[k_{cat.}/K_m(X)]/[k_{cat.}/K_m$ (X = Z-Gly)].

	Pepsin		Cathepsin D	
X	Pig	Pig	Bovine	Chicken liver
Z-Gly	1.00	1.00	1.00	1.00
Z-Ala	—	4.38	—	2.06
Z-Gly-Gly	19.9	1.25	2.00	1.02
Z-Gly-Ala	432	11.8	30.8	15.6
Z-Ala-Ala	819	9.97	53.8	23.6

of the enzyme. The proteolytic activity of cathepsin D is known to be enhanced by other small molecules such as glycine ethyl ester (Dionyssiou-Asteriou & Rakitzis, 1979) and polyphosphate anions (Watabe *et al.*, 1979).

The kinetic parameters for the hydrolysis of neutral substrates by pepsin and cathepsin D provide further information on the specificity of these enzymes. In the following discussion these substrates, which are mostly based on the sequence -Phe(NO_2)-Trp-$NHCH_2CH_2OH$, are compared with those cationic substrates (Table 4) studied by Fruton and his associates (see Sampath-Kumar & Fruton, 1974) and which are based on the sequence -Phe-Phe-OR'' [R'' = 3-(4-pyridyl)propyl]. Examination of Tables 2 and 3 reveals that the presence of residues at sites P_2 and P_3, in the notation of Schechter & Berger (1967), makes substrates more susceptible to cleavage by both enzymes. Alanine is considerably more effective in this role at site P_2 than is glycine. These findings are in agreement with those obtained by Fruton and his associates (see Sampath-Kumar & Fruton, 1974). For both enzymes the presence of a single alanine residue at site P_2 is more effective than glycine residues at sites P_2 and P_3. This finding must, however, be considered in the light of the results shown in Table 4, that Z-Gly-Phe-Phe-OR'' is a much poorer substrate for pepsin than is Z-Gly-Gly-Phe-Phe-OR''. The results from the current study do not fit this pattern, since a glycine residue at site P_2 seems equally effective as glycine residues at sites P_2 and P_3. Considering pepsin firstly and comparing each of the neutral substrates with the corresponding cationic substrate, Z-Phe-Phe-OR'' (Sampath-Kumar & Fruton, 1974) and Z-Gly-Phe-Phe-OR'' have similar specificity constants (3500 $M^{-1}\cdot s^{-1}$ and 8700 $M^{-1}\cdot s^{-1}$ respectively) to those for Z-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ and Z-Gly-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ (3510 $M^{-1}\cdot s^{-1}$ and 13100 $M^{-1}\cdot s^{-1}$ respectively). The better cationic substrates Z-Gly-Gly-Phe-Phe-OR'', Z-Gly-Ala-Phe-Phe-OR'' and Z-Ala-Ala-Phe-Phe-OR'' have very much greater specificity constants (171000 $M^{-1}\cdot s^{-1}$, 3720000 $M^{-1}\cdot s^{-1}$ and 7050000 $M^{-1}\cdot s^{-1}$ respectively) than do Z-Gly-Gly-Phe(NO_2)-Trp-$NHCH_2CH_2OH$, Z-Gly-Ala-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ and Z-Ala-Ala-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ (16300 $M^{-1}\cdot s^{-1}$, 155000 $M^{-1}\cdot s^{-1}$ and 131000 $M^{-1}\cdot s^{-1}$ respectively). The greater specificity constants exhibited by the cationic substrates are largely the result of $k_{cat.}$ values that are about 10 times higher than the $k_{cat.}$ values of the neutral substrates. This may be due to the presence of 2-methoxyethanol as a solubilizing agent for the neutral substrates. For example, the specificity constant for the hydrolysis of substrate (XIX) in water is similar to that of substrate (XI) in aqueous 2-methoxyethanol. On the other hand, we have shown that 1% (v/v) 2-methoxyethanol slightly decreases the rate of hydrolysis of Z-His-Phe(NO_2)-Phe-OMe, and Cornish-Bowden & Knowles (1969) have shown a similar effect on the rate of peptic hydrolysis of *N*-Ac-Phe-Phe-OH by NN-dimethylformamide (1.2%, v/v). It is possible that the better substrates containing more amino acids, at least two of which have hydrophobic side chains, adopt in solution a preferred conformation that is influenced markedly by organic solvents. Alternatively, 2-methoxyethanol may cause changes in the conformation of the enzyme–substrate complex. The specificity constants for hydrolysis of the neutral substrates by cathepsin D, however, are in all cases greater than those exhibited by the corresponding cationic substrate by about 4–8-fold. Comparison of these results is more complex for cathepsin D than for pepsin because cathepsin D from different sources was used in each of the two studies. It would appear, however, that 2-methoxyethanol causes less inhibition of cathepsin D-catalysed hydrolysis than of pepsin-catalysed hydrolysis, or alternatively the charge of the cationic group has a greater inhibitory effect with cathepsin D than with pepsin. The presence of a free α-amino group in the substrate Ala-Phe(NO_2)-Trp-$NHCH_2CH_2OH$ causes a marked decrease in the specificity constant of pepsin compared with the *N*-benzyloxycarbonyl-protected peptide. This peptide was not hydrolysed at all by cathepsin D.

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