Kinetics and Mechanism of Catalysis by Proteolytic Enzymes

THE KINETICS OF HYDROLYSIS OF DERIVATIVES OF L-LYSINE AND S-(β -AMINOETHYL)-L-CYSTEINE (THIALYSINE) BY BOVINE TRYPSIN

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1. Several esters of the α -N-toluene-*p*-sulphonyl and N-benzoyl derivatives of L-lysine and S-(β -aminoethyl)-L-cysteine have been synthesized. 2. The kinetics of hydrolysis of the esters by bovine trypsin have been compared. Values of k_0 are similar for corresponding derivatives of the isosteric amino acids and deacylation of an acyl-enzyme appears to be rate-determining in each case. There are, however, some quantitative kinetic differences between the various series of substrates.

The present work is a continuation of a systematic study of the specificity and mechanism of action of trypsin and related enzymes (Baines, Baird & Elmore, 1964; Curragh & Elmore, 1964; Baird, Curragh & Elmore, 1965). Derivatives of lysine are well-known substrates for these enzymes and we have accordingly synthesized and studied the kinetics of hydrolysis by trypsin of esters of α -N-toluene-*p*-sulphonyl- and α -N-benzoyl-L-lysine (Ia and Ib). It has been shown that cysteine residues in proteins can be converted into S-(β -aminoethyl)-L-cysteine by reaction with 2-bromoethylamine hydrobromide or ethyleneimine (Lindley, 1956, 1959; Raftery & Cole, 1963) and that the peptide bonds involving the carbonyl group of the latter amino acid are hydrolysed by trypsin. Since S-(β -aminoethyl)-L-cysteine is an isostere of L-lysine, we have synthesized esters of S-(β -aminoethyl)-N-toluene-*p*-sulphonyl-L-cysteine (IIa) and

-N-benzoyl-L-cysteine (IIb) and we have studied the kinetics of their hydrolysis by trypsin for comparison. The results of some of this work have been briefly reported by Elmore, Baird, Roberts & Smyth (1964).

EXPERIMENTAL

Materials

Trypsin, twice-crystallized, salt-free (Worthington Biochemicals Corp., Freehold, N.J., U.S.A.), was dialysed against mN-HCl, centrifuged and freeze-dried. N-Toluene-*p*-sulphonyl-L-cysteine was prepared from NN'-ditoluene-*p*-sulphonyl-L-cysteine (Bloch & Clarke, 1938) by the method of Dadic, Fles & Markovac (1961). S-(β -Benzoyloxycarbonylaminoethyl)-L-cysteine was prepared by the method of Lindley (1959) although our product had m.p. 216–218° (decomp.) compared with the recorded m.p. 204–205°. ϵ -N-Benzoyloxycarbonyl- α -N-toluene-*p*-sulphonyl-L-lysine, α -N-toluene-*p*-sulphonyl-L-lysine and its

(Ia; R = CH₃, CH₃·CH₂, CH₃·CH₂·CH₂, β -C₁₀H₇ or 3-C₅H₄N, HBr, R' = *p*-CH₃·C₆H₄·SO₂, X⁻ = Br⁻ or Cl⁻)

(Ib; R = CH₃ or C₆H₁₁, R' = C₆H₅·CO, X⁻ = *p*-CH₃·C₆H₄·SO₃⁻)

(IIa; R = CH₃, CH₃·CH₂ or CH₃·CH₂·CH₂, R' = *p*-CH₃·C₆H₄·SO₂, X⁻ = Cl⁻)

(IIb; R = CH₃ or C₆H₁₁, R' = C₆H₅·CO, X⁻ = Cl⁻ or *p*-CH₃·C₆H₄·SO₃⁻)

methyl ester hydrochloride, which had $[\alpha]_D^{20} -10.3 \pm 0.3^\circ$ (c 5 in water), were prepared as described by Barrass & Elmore (1957a,b). β -Naphthol was purified by zone-melting when it had m.p. 121–122°; di- β -naphthyl sulphite, m.p. 79°, was prepared by the method of Richter (1916).

Alkyl esters of ϵ -N-benzoyloxycarbonyl- α -N-toluene-*p*-sulphonyl-L-lysine. ϵ -N-Benzoyloxycarbonyl- α -N-toluene-*p*-sulphonyl-L-lysine ethyl ester, having m.p. 100–102° (Found: C, 59.7; H, 6.8; N, 6.1. $C_{23}H_{30}N_2O_6S$ requires C, 59.7; H, 6.5; N, 6.1%), and the corresponding *n*-propyl ester, having m.p. 96° (Found: C, 60.3; H, 6.8; N, 6.2. $C_{24}H_{32}N_2O_6S$ requires C, 60.5; H, 6.8; N, 5.9%), were synthesized in 85% yield by the method used for the methyl ester (Barrass & Elmore, 1957b).

ϵ -N-Benzoyloxycarbonyl- α -N-toluene-*p*-sulphonyl-L-lysine 3-pyridyl ester. A solution of ϵ -N-benzoyloxycarbonyl- α -N-toluene-*p*-sulphonyl-L-lysine (10 m-moles) and 3-hydroxypyridine (10 m-moles) in tetrahydrofuran (20 ml.) was treated with *NN'*-dicyclohexylcarbodi-imide (10.5 m-moles) and left overnight. *NN'*-Dicyclohexylurea was filtered off, solvent was removed under reduced pressure and the residual oil was dissolved in ethyl acetate. The solution was washed with ice-cold saturated $NaHCO_3$ solution, then with water and dried. After removal of solvent under reduced pressure, the product (65%) was crystallized from methylene chloride–light petroleum (b.p. 60–80°), when it had m.p. 96° (Found: C, 61.1; H, 5.6; N, 8.1. $C_{26}H_{26}N_3O_6S$ requires C, 61.0; H, 5.7; N, 8.2%). This compound was also synthesized from tetraethyl pyrophosphite (Anderson, Blodinger & Welcher, 1952) in place of *NN'*-dicyclohexylcarbodi-imide.

Alkyl ester hydrochlorides of α -N-toluene-*p*-sulphonyl-L-lysine. Two general methods were used to prepare these compounds.

(a) Alkyl esters of ϵ -N-benzoyloxycarbonyl- α -N-toluene-*p*-sulphonyl-L-lysine were hydrogenolysed at atmospheric pressure in aqueous ethanol containing 1 equiv. of HCl in the presence of 10% palladium on charcoal. After the uptake of hydrogen had ceased, the solution was filtered and evaporated under reduced pressure. The ester hydrochlorides (95%) were crystallized from propan-2-ol–ether.

(b) α -N-Toluene-*p*-sulphonyl-L-lysine was esterified by using the requisite alcohol saturated with dry HCl. The product (85%) was obtained by evaporation of the solution under reduced pressure and crystallization of the residue from propan-2-ol–ether.

Obtained by these methods were: α -N-toluene-*p*-sulphonyl-L-lysine ethyl ester hydrochloride (Ia; $R=CH_3 \cdot CH_2$, $X=Cl$), having m.p. 179°, $[\alpha]_D^{20} -6.4 \pm 0.2^\circ$ (c 5 in water) (Found: C, 49.7; H, 6.7; N, 7.9. $C_{15}H_{25}ClN_2O_4S$ requires C, 49.4; H, 6.9; N, 7.7%); α -N-toluene-*p*-sulphonyl-L-lysine *n*-propyl ester hydrochloride (Ia; $R=CH_3 \cdot CH_2 \cdot CH_2$, $X=Cl$), having m.p. 149°, $[\alpha]_D^{20} -4.2 \pm 0.2^\circ$ (c 5 in water) (Found: C, 50.5; H, 7.2; N, 7.5. $C_{16}H_{27}ClN_2O_4S$ requires C, 50.7; H, 7.2; N, 7.4%).

α -N-Toluene-*p*-sulphonyl-L-lysine 3-pyridyl ester hydrobromide (Ia; $R=3-C_6H_4N, HBr$, $X=Br$). ϵ -N-Benzoyloxycarbonyl- α -N-toluene-*p*-sulphonyl-L-lysine 3-pyridyl ester was treated with an excess of dry HBr in nitromethane at room temperature. After 20 min., a large excess of dry ether was added and the product separated as an oil. This was dissolved in boiling propan-2-ol, and ether was added to the cooled solution. The crystalline precipitate was centrifuged, washed with dry ether and recrystallized twice from propan-2-ol–ether. The ester dihydrobromide (60%) was

very hygroscopic and was stored in a vacuum desiccator (Found: C, 39.9; H, 4.9; N, 8.0; Br, 29.4. $C_{18}H_{25}Br_2N_3O_4S$ requires C, 40.1; H, 4.7; N, 7.8; Br, 29.6%).

α -N-Toluene-*p*-sulphonyl-L-lysine β -naphthyl ester hydrobromide (Ia; $R=\beta-C_{10}H_7$, $X=Br$). A mixture of α -N-benzoyloxycarbonyl- α -N-toluene-*p*-sulphonyl-L-lysine (4.34 g.) and dinaphthyl sulphite (4.0 g.) in dry pyridine (10 ml.) was allowed to stand overnight. After removal of pyridine under reduced pressure, the residual oil was dissolved in ethyl acetate and the solution was washed successively with ice-cold *N*-HCl, water, saturated $NaHCO_3$ solution, water and then dried. Solvent was removed under reduced pressure and the residual oil was treated with a slight excess of dry HBr in acetic acid. After 20 min., a large excess of dry ether was added. The supernatant liquid was decanted and the residual oil was crystallized from propan-2-ol–ether. After two further recrystallizations, the ester hydrobromide (70%) had m.p. 184°, $[\alpha]_D^{20} -13.3 \pm 0.2^\circ$ (c 5 in methanol) (Found: C, 54.1; H, 5.5; N, 5.5. $C_{23}H_{27}BrN_2O_4S$ requires C, 54.4; H, 5.4; N, 5.5%).

ϵ -N-Benzoyloxycarbonyl- α -N-benzoyl-L-lysine. ϵ -N-Benzoyloxycarbonyl-L-lysine (30 m-moles) was treated in aqueous solution with benzoyl chloride (45 m-moles) at 0° and the pH was kept at 9.5 by the addition of 2 *N*-NaOH from a pH-stat. When benzylation was complete, the alkaline solution was extracted with ethyl acetate and then acidified with 10 *N*-HCl. The resulting oil, after extraction into ethyl acetate, crystallized on addition of light petroleum (b.p. 60–80°). The product (77%) was freed from benzoic acid by extraction with hot light petroleum (b.p. 60–80°) in a Soxhlet thimble and recrystallized from ethyl acetate–light petroleum, when it had m.p. 108–110° (Found: C, 65.7; H, 6.3; N, 7.2. $C_{21}H_{24}N_2O_5$ requires C, 65.6; H, 6.3; N, 7.3%).

Esters of ϵ -N-benzoyloxycarbonyl- α -N-benzoyl-L-lysine. The methyl and cyclohexyl esters were obtained by the same method as that used to synthesize alkyl esters of ϵ -N-benzoyloxycarbonyl- α -N-toluene-*p*-sulphonyl-L-lysine. The methyl ester crystallized from ethyl acetate–light petroleum (b.p. 60–80°) and had m.p. 74–75° [Izumiyama, Okazaki, Matsumoto & Takiguchi (1959) quote m.p. 74–76°]. The cyclohexyl ester (76%) had m.p. 99–100° (Found: C, 69.7; H, 7.4; N, 5.9. $C_{27}H_{34}N_2O_5$ requires C, 69.5; H, 7.3; N, 6.0%).

α -N-Benzoyl-L-lysine methyl ester toluene-*p*-sulphonate (Ib; $R=CH_3$). ϵ -N-Benzoyloxycarbonyl- α -N-benzoyl-L-lysine methyl ester (1 g.) in ethanol (25 ml.) was hydrogenolysed in the presence of toluene-*p*-sulphonic acid (0.5 g.) and 10% palladium on charcoal catalyst (0.25 g.). After filtration, the product (72%) crystallized on the addition of ether and was recrystallized from ethanol–ether, when it had m.p. 100–101°, $[\alpha]_D^{20} +13.3 \pm 0.1^\circ$ (c 3 in ethanol) (Found: C, 56.6; H, 6.6; N, 5.9. $C_{21}H_{28}N_2O_6S \cdot \frac{1}{2}H_2O$ requires C, 56.6; H, 6.6; N, 6.3%).

α -N-Benzoyl-L-lysine cyclohexyl ester toluene-*p*-sulphonate (Ib; $R=C_6H_{11}$). Prepared in a manner similar to that described for the methyl ester, the cyclohexyl ester (69%) had m.p. 124–126°, $[\alpha]_D^{20} +9.36 \pm 0.1^\circ$ (c 4 in ethanol) (Found: C, 61.1; H, 7.0; N, 5.4. $C_{26}H_{36}N_2O_6S \cdot \frac{1}{2}H_2O$ requires C, 60.8; H, 7.3; N, 5.5%). Attempts to make the hydrochlorides, hydrobromides or hydrogen sulphates of these esters gave only oily products.

S-(β -Aminoethyl)-N-toluene-*p*-sulphonyl-L-cysteine. To a solution of N-toluene-*p*-sulphonyl-L-cysteine (2.75 g.) in

saturated NaHCO_3 solution (50ml.) was added ethyleneimine (0.5g.) with mechanical stirring. The product started to crystallize after a few minutes. When a test portion of the reaction mixture no longer gave a positive reaction for thiol with sodium nitroprusside, the pH was adjusted to 7 and the solid was collected. It was redissolved in dilute HCl and the solution was extracted to remove traces of NN' -ditoluene- p -sulphonyl-L-cysteine formed by oxidation during the reaction. After neutralization of the solution to pH 7, the S -(β -aminoethyl)- N -toluene- p -sulphonyl-L-cysteine (85%) was filtered off, washed successively with water, acetone and ether and then dried, when it had m.p. 254–256° (decomp.) (Found: C, 44.9; H, 5.5; N, 8.9. $\text{C}_{12}\text{H}_{18}\text{N}_2\text{O}_4\text{S}_2$ requires C, 45.3; H, 5.7; N, 8.8%).

Alkyl ester hydrochlorides of S -(β -aminoethyl)- N -toluene- p -sulphonyl-L-cysteine. These compounds were obtained by method (b) described above for the corresponding derivatives of α - N -toluene- p -sulphonyl-L-lysine. They were recrystallized first from a mixture of the alcohol corresponding to the ester and ether, then from propan-2-ol-ether. The *methyl ester hydrochloride* (IIa; $\text{R}=\text{CH}_3$) (75%) had m.p. 127–128°, $[\alpha]_D^{25} -22.6 \pm 0.2^\circ$ (c 2.37 in ethanol) (Found: C, 42.1; H, 5.8; N, 7.8. $\text{C}_{13}\text{H}_{21}\text{ClN}_2\text{O}_4\text{S}_2$ requires C, 42.3; H, 5.7; N, 7.6%); the *ethyl ester hydrochloride* (IIa; $\text{R}=\text{CH}_3\cdot\text{CH}_2$) (80%) had m.p. 144–145°, $[\alpha]_D^{25} -23.5 \pm 0.3^\circ$ (c 4 in ethanol) (Found: C, 43.7; H, 6.0; N, 7.3. $\text{C}_{14}\text{H}_{23}\text{ClN}_2\text{O}_4\text{S}_2$ requires C, 43.9; H, 6.1; N, 7.3%); the *n-propyl ester hydrochloride* (IIa; $\text{R}=\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}_2$) (78%) had m.p. 140.5–141.5°, $[\alpha]_D^{25} -22.8 \pm 0.2^\circ$ (c 4 in ethanol) (Found: C, 45.6; H, 6.5; N, 7.0. $\text{C}_{15}\text{H}_{25}\text{ClN}_2\text{O}_4\text{S}_2$ requires C, 45.4; H, 6.4; N, 7.1%).

β -(β -Aminoethylsulphonyl)- N -toluene- p -sulphonyl-L-alanine. S -(β -Aminoethyl)- N -toluene- p -sulphonyl-L-cysteine (4m-moles), dissolved in water (7.5ml.), was treated with 5 N - HClO_4 (1.25ml.), 0.5 M -ammonium molybdate (0.5ml.) and H_2O_2 (15m-moles). The mixture was stirred at room temperature for 24 hr., acidified to pH 1 with HCl and evaporated to dryness under reduced pressure. The residue was dissolved in a small volume of water and caused to crystallize by adjusting the solution to pH 7. The *sulphone* (61%) was recrystallized from hot water, when it had m.p. 205–207° (decomp.) (Found: C, 41.3; H, 5.3; N, 8.0. $\text{C}_{12}\text{H}_{18}\text{N}_2\text{S}_2\text{O}_6$ requires C, 41.1; H, 5.2; N, 8.0%).

β -(β -Aminoethylsulphonyl)- N -toluene- p -sulphonyl-L-alanine methyl ester hydrochloride. The foregoing acid was esterified with methanol saturated with dry HCl. After recrystallization from methanol-ether and then propan-2-ol-ether, the *product* (66%) had m.p. 139–141° (Found: C, 36.9; H, 5.5; N, 6.6. $\text{C}_{13}\text{H}_{21}\text{ClN}_2\text{O}_6\text{S}_2\cdot\text{H}_2\text{O}$ requires C, 37.3; H, 5.5; N, 6.7%).

S -(β -Benzoyloxycarbonylaminoethyl)- N -benzoyl-L-cysteine. S -(β -Benzoyloxycarbonylaminoethyl)-L-cysteine (3g.) was suspended in a mixture of ethanol (110ml.) and water (60ml.) at 0° and the pH was adjusted to 11. Benzoyl chloride (2.8g.) was added dropwise and the pH was maintained constant by means of a pH-stat. After reaction was complete (negative ninhydrin reaction), the solution was adjusted to pH 7, evaporated under reduced pressure and then acidified. The product was extracted with ethyl acetate and the extract was washed with water and dried (MgSO_4). Addition of cyclohexylamine caused the *cyclohexylammonium salt* (61%) to crystallize, which had m.p. 112–114° after recrystallization from ethanol-ether (Found: C, 62.4; H, 7.0; N, 8.2. $\text{C}_{26}\text{H}_{35}\text{N}_3\text{O}_5\text{S}$ requires C, 62.2; H,

7.0; N, 8.4%). Attempts to carry out the benzylation in aqueous solution or mixtures of water and aprotic organic solvents gave large amounts of benzoic acid that prevented crystallization of the product. In the presence of ethanol, ethyl benzoate is formed which does not interfere with the isolation of the product.

S -(β -Aminoethyl)- N -benzoyl-L-cysteine. The cyclohexylammonium salt of S -(β -benzyloxycarbonylaminoethyl)- N -benzoyl-L-cysteine (6.5g.) was dissolved in water and acidified. The free acid was extracted with ethyl acetate, and the extract was dried (MgSO_4) and evaporated. Freshly prepared 45% HBr in acetic acid (9ml.) was added, the solution was gently warmed and evaporated under reduced pressure. The residual oil was triturated with ether and the washings were decanted. The oil was dissolved in ethanol and tri- n -butylamine was added dropwise until the solution was alkaline to moist phenol-red indicator paper. S -(β -Aminoethyl)- N -benzoyl-L-cysteine (56%) was precipitated by the addition of ether and recrystallized by suspension in hot ethanol with dropwise addition of water until it dissolved. Crystallization began on cooling and was completed by addition of ether. The acid had m.p. 224–225° (decomp.) (Found: C, 53.4; H, 5.9; N, 11.0. $\text{C}_{12}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$ requires C, 53.7; H, 6.0; N, 10.4%).

S -(β -Aminoethyl)- N -benzoyl-L-cysteine methyl ester hydrochloride (IIb; $\text{R}=\text{CH}_3$, $\text{X}=\text{Cl}$). This ester (72%) was prepared by method (b) described above for esters of α - N -toluene- p -sulphonyl-L-lysine. Recrystallized from ethanol-ether, it had m.p. 160–162°, $[\alpha]_D^{20} +58.7 \pm 0.1^\circ$ (c 3.2 in ethanol) (Found: C, 48.6; H, 6.0; N, 8.5. $\text{C}_{13}\text{H}_{19}\text{ClN}_2\text{O}_3\text{S}$ requires C, 49.0; H, 6.0; N, 8.8%).

S -(β -Aminoethyl)- N -benzoyl-L-cysteine cyclohexyl ester toluene- p -sulphonate (IIb; $\text{R}=\text{C}_6\text{H}_{11}$, $\text{X}=\text{p-CH}_3\cdot\text{C}_6\text{H}_4\cdot\text{SO}_3^-$). S -(β -Aminoethyl)- N -benzoyl-L-cysteine (1.62g.) was azeotropically distilled with a mixture of cyclohexanol and benzene for 5 hr. in the presence of toluene- p -sulphonic acid (2.3g.). The product (63%) was precipitated by the addition of a large volume of ether and was recrystallized from propan-2-ol-ether, when it had m.p. 80–82°, $[\alpha]_D^{20} +27.5 \pm 0.1^\circ$ (c 4 in ethanol) (Found: C, 57.1; H, 6.8; N, 5.7. $\text{C}_{25}\text{H}_{34}\text{N}_2\text{O}_6\text{S}_2$ requires C, 57.4; H, 6.6; N, 5.4%).

Kinetic and computational techniques

Hydrolysis of esters was followed by means of a pH-stat and recorder (Baines *et al.* 1964). Kinetic and activation constants were computed by statistical methods described by Elmore, Kingston & Shields (1963). Some of the values of k_0 used for computing activation constants were obtained from determinations of K_m and some were obtained from single runs. The technique of determining k_0 from single runs was only used at temperatures between those at which K_m had been determined. From the kinetic behaviour at nearby temperatures, it was possible to decide what concentration of substrate was appropriate to attain maximum velocity and to avoid, for N -toluene- p -sulphonyl derivatives, substrate activation. The hydrolysis of α - N -toluene- p -sulphonyl-L-lysine β -naphthyl ester was also followed spectrophotometrically by using an Optica CF4 single-beam recording spectrophotometer. The composition of the reaction medium was as follows: H_3BO_3 (8.75 mM), $\text{Na}_2\text{B}_4\text{O}_7$ (3.75 mM), NaCl (0.1 M) and CaCl_2 (2 mM) and its pH was 8.01 at 25°. The progress of the reaction was

followed by the increase in $E_{328m\mu}$; the ester and α -*N*-toluene-*p*-sulphonyl-L-lysine are virtually transparent at this wavelength, whereas β -naphthol has ϵ 1750.

RESULTS AND DISCUSSION

Kinetic constants for the hydrolysis of esters of α -*N*-toluene-*p*-sulphonyl-L-lysine and *S*-(β -aminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine by trypsin are listed in Table 1. Kinetic constants for the hydrolysis of the corresponding *N*-benzoyl derivatives are given in Table 2. Values of k_0 appear to be independent of the nature of the ester group within each series. This kind of behaviour has now been observed for eight series of ester substrates of trypsin (Schwert & Eisenberg, 1949; Baines *et al.* 1964; Baird *et al.* 1965) and strongly supports the view that the mechanism of catalysis by trypsin involves acylation of the enzyme by the substrate followed by a slower deacylation step. Values of k_0 for each pair of series of esters with the same α -*N*-substituent are not greatly different. This is to be expected, since the distance between the protonated amino group and the susceptible ester bond probably does not differ by more than 0.3 Å if the

side chains are both in their most-favoured conformation. In contrast, values of k_0 for the hydrolysis of the methyl esters of α -*N*-hippuryl-L-lysine and α -*N*-(*N*-phthaloylglycyl)-L-lysine are 6–8 times as great as for the corresponding derivatives of *S*-(β -aminoethyl)-L-cysteine (Hermann & Gründig, 1963; Tesser, Nivard & Gruber, 1964). The values of k_0 for the present compounds also do not differ greatly from those of the corresponding arginine derivatives, which presumably have side chains of similar length.

Unfortunately, k_0 could not be determined for the 3-pyridyl ester of α -*N*-toluene-*p*-sulphonyl-L-lysine. Apart from the difficulty of handling it owing to its hygroscopic nature, it was hydrolysed too quickly in the absence of enzyme to serve as a useful substrate. The β -naphthyl ester, on the other hand, is not hygroscopic, is relatively stable to solvolysis, shows no strong tendency to lactamize at the pH values used for kinetic studies with trypsin and is likely to be a useful addition to the range of specific substrates available for trypsin and related enzymes. Its hydrolysis by trypsin can be followed conveniently spectrophotometrically.

Esters of α -*N*-toluene-*p*-sulphonyl-L-lysine have

Table 1. k_0 and K_m values for the trypsin-catalysed hydrolysis of esters of α -*N*-toluene-*p*-sulphonyl-L-lysine and *S*-(β -aminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine at pH 8.0

Values given are means $\pm$ S.D.

Substrate	Temp.	k_0 (sec. ⁻¹)	$10^5 K_m$ (M)	$10^5 [S]_0$ (M)	$10^9 [E]_0$ (M)
(Ia; R = CH ₃ , X ⁻ = Cl ⁻)	15°	40.9 $\pm$ 0.9	2.12 $\pm$ 0.20	2.0–70.0	10.5
	20	52.3 $\pm$ 2.8	3.38 $\pm$ 0.36	2.0–70.0	9.61
	25	67.2 $\pm$ 2.4	4.18 $\pm$ 0.43	2.0–70.0	7.52
	30	92.7 $\pm$ 1.6	6.01 $\pm$ 0.41	2.0–70.0	7.70
	37	129.3 $\pm$ 2.2	8.26 $\pm$ 0.36	2.0–70.0	7.41
(Ia; R = CH ₃ ·CH ₂ , X ⁻ = Cl ⁻)	25	67.8 $\pm$ 1.8	3.63 $\pm$ 0.40	2.0–70.0	6.84
(Ia; R = CH ₃ ·CH ₂ ·CH ₂ , X ⁻ = Cl ⁻)	25	69.2 $\pm$ 3.0	2.21 $\pm$ 0.20	2.0–70.0	7.35
(Ia; R = β -C ₁₀ H ₇ , X ⁻ = Br ⁻)	25	67.6 $\pm$ 0.8	0.78 $\pm$ 0.24	0.0414–2.90	4.38
(IIa; R = CH ₃)	15	27.4 $\pm$ 0.5	—	—	—
	20	38.9 $\pm$ 0.4	13.3 $\pm$ 0.5	2.0–240.0	8.00–8.19
	25	54.8 $\pm$ 0.6	21.6 $\pm$ 0.7	6.0–300.0	7.72
	30	79.5 $\pm$ 0.4	32.9 $\pm$ 0.4	5.0–240.0	8.65
	37	123.6 $\pm$ 0.9	44.4 $\pm$ 0.8	5.0–300.0	7.52
(IIa; R = CH ₃ ·CH ₂)	25	55.6 $\pm$ 1.1	36.9 $\pm$ 1.4	5.0–200.0	9.84
(IIa; R = CH ₃ ·CH ₂ ·CH ₂)	25	56.7 $\pm$ 2.0	20.5 $\pm$ 1.6	6.0–200.0	9.26

Table 2. k_0 and K_m values for the trypsin-catalysed hydrolysis of esters of α -*N*-benzoyl-L-lysine and *S*-(β -aminoethyl)-*N*-benzoyl-L-cysteine at pH 8.0 and 25°

Values given are means $\pm$ S.D.

Substrate	k_0 (sec. ⁻¹)	$10^5 K_m$ (M)	$10^5 [S]_0$ (M)	$10^9 [E]_0$ (M)
(Ib; R = CH ₃)	16.6 $\pm$ 0.1	1.67 $\pm$ 0.04	0.7–90.0	11.0
(Ib; R = C ₆ H ₁₁)	16.5 $\pm$ 0.2	0.658 $\pm$ 0.056	1.0–50.0	6.75
(IIb; R = CH ₃ , X ⁻ = Cl ⁻)	20.6 $\pm$ 0.4	9.41 $\pm$ 0.63	2.0–400.0	14.1
(IIb; R = C ₆ H ₁₁ , X ⁻ = <i>p</i> -CH ₃ ·C ₆ H ₄ ·SO ₃ ⁻)	22.0 $\pm$ 0.2	2.95 $\pm$ 0.11	0.8–100.0	6.60

K_m values similar in magnitude to those of esters of α -*N*-toluene-*p*-sulphonyl-L-arginine (Baines *et al.* 1964), but with the present substrates K_m decreases with increasing size of the ester group. With the β -naphthyl ester, part of this effect may stem from an increase in k_{+2} , since aryl esters are better acylating agents than alkyl esters. This explanation, however, cannot account for the smaller decrease in K_m as the ester group is increased in size from methyl to propyl, since k_{+2} would be expected to decrease and consequently K_m would increase (Curragh & Elmore, 1964). The decrease in K_m probably results from an increase in k_{+1} , a decrease in k_{-1} or both. Stabilization of the Michaelis complex might result from the increased possibility of hydrophobic bonding with the bigger ester groups. Alternatively, k_{+2} might actually be increased, contrary to expectation, owing to induced conformational changes in the enzyme that assist the acylation step (Koshland, 1958). The values of K_m for esters of *S*-(β -aminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine probably follow a similar trend, although the ethyl ester has a slightly higher K_m than the methyl or propyl ester. It is possible that this reflects a competition between two opposing effects,

the one being a decrease in k_{+2} with increasing size of the alkyl group leading to an increase in K_m , the other being a stabilization of the Michaelis complex owing to increased possibilities of hydrophobic bonding as the size of the alkyl group is increased. Values of K_m for this series of esters are about 5–10 times as great as for esters of α -*N*-toluene-*p*-sulphonyl-L-lysine. There is no obvious explanation for this difference. It is noteworthy that, at high concentrations of substrate, the velocities of hydrolysis of both series of esters bearing the

Table 3. Apparent p*K* values for the group(s) involved in the trypsin-catalysed hydrolysis of methyl esters of derivatives of L-lysine and *S*-(β -aminoethyl)-L-cysteine

Values given are means $\pm$ s.d.

Substrate	p <i>K</i> _(app.) at 25°
(Ia; R=CH ₃ , X ⁻ =Cl ⁻)	7.38 $\pm$ 0.02
(IIa; R=CH ₃)	7.05 $\pm$ 0.01
(Ib; R=CH ₃)	6.29 $\pm$ 0.02
(IIb; R=CH ₃ , X ⁻ =Cl ⁻)	6.16 $\pm$ 0.02

Fig. 1. Lineweaver-Burk plot for the trypsin-catalysed hydrolysis of *S*-(β -aminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine *n*-propyl ester (IIa; R=CH₃·CH₂·CH₂) at pH 8.0 and 25°.

Table 4. *Activation constants for the trypsin-catalysed hydrolysis of methyl ester derivatives of L-lysine and S-(β-aminoethyl)-L-cysteine*Values given are means $\pm$ S.D.

Substrate	Energy of activation (kcal./mole)	Enthalpy of activation (kcal./mole at 25°)	Entropy of activation (e.u. at 25°)
(Ia; R = CH ₃ , X ⁻ = Cl ⁻)	9.37 $\pm$ 0.21 (5)*	8.78 $\pm$ 0.21	-20.6 $\pm$ 0.7
(IIa; R = CH ₃)	11.88 $\pm$ 0.07 (15)	11.29 $\pm$ 0.07	-12.6 $\pm$ 0.2
(Ib; R = CH ₃)	10.73 $\pm$ 0.31 (28)	10.13 $\pm$ 0.31	-19.0 $\pm$ 1.0
(IIb; R = CH ₃ , X ⁻ = Cl ⁻)	9.66 $\pm$ 0.25 (18)	9.07 $\pm$ 0.25	-21.8 $\pm$ 0.8

* Number of values of k_0 used for computing activation constants is given in parentheses. Measurements were made at 5–11 different temperatures.

α -N-toluene-*p*-sulphonyl group were increased above the maximum velocity expected for Michaelis-Menten kinetics. A Lineweaver-Burk plot thus became non-linear at low values of $1/[S]$ (Fig. 1). This behaviour has previously been observed in the hydrolysis of α -N-toluene-*p*-sulphonyl-L-arginine methyl ester by trypsin and thrombin (Trowbridge, Krehbiel & Laskowski, 1963; Baines *et al.* 1964; Curragh & Elmore, 1964; Bechet, 1964; Bechet & Yon, 1964) and has been ascribed to activation by the substrate. In an attempt to determine the effect on K_m of a polar group in the side chain, *S*-(β-aminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine was oxidized to the corresponding sulphone, β-(β-aminoethylsulphonyl)-*N*-toluene-*p*-sulphonyl-L-alanine. Unfortunately, the methyl ester of the latter compound was rapidly hydrolysed in aqueous solution at pH 7 and could not therefore be used as a substrate.

Values of K_m for substrates bearing the α -*N*-benzoyl group are rather lower than for the α -*N*-toluene-*p*-sulphonyl derivatives. Aside from this, the K_m values follow a similar pattern: increase in the size of the ester group decreases K_m in both series and values of K_m for derivatives of *S*-(β-aminoethyl)-*N*-benzoyl-L-cysteine are approx. 5 times those of the corresponding derivatives of α -*N*-benzoyl-L-lysine.

Values of k_0 for the foregoing esters have been determined under a variety of conditions. Change of ionic strength had no effect. This behaviour has now been observed for a number of reactions catalysed by trypsin and thrombin where deacylation is apparently rate-determining.

The variation of k_0 with pH led to linear plots of $1/v$ against $[H^+]$ from which values of $pK_{(app.)}$ were computed (Table 3). Values of $pK_{(app.)}$ for the hydrolysis of substrates containing the α -*N*-benzoyl group were close to that reported for the trypsin-catalysed hydrolysis of α -*N*-benzoyl-L-arginine ethyl ester (Gutfreund, 1955). Values of $pK_{(app.)}$ for α -*N*-toluene-*p*-sulphonyl derivatives,

however, were considerably higher and comparable with values determined for α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester, α -*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester and methyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate (Baines *et al.* 1964; Baird *et al.* 1965).

From the variation of k_0 with temperature, activation constants were computed and are recorded in Table 4. It is difficult to discern any relationship between the structure of a substrate and the activation constants for its hydrolysis. For example, the energy of activation for the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-lysine methyl ester is lower than that for the hydrolysis of *S*-(β-aminoethyl)-*N*-toluene-*p*-sulphonyl-L-cysteine methyl ester, whereas the converse is true for the corresponding α -*N*-benzoyl derivatives. From the limited number of derivatives so far examined, however, there is an indication that the entropy of activation is more negative and the energy of activation is lower for the hydrolysis of derivatives of lysine compared with those of arginine (cf. Baines *et al.* 1964).

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REFERENCES

- Anderson, G. W., Blodinger, J. & Welcher, D. (1952). *J. Amer. chem. Soc.* **74**, 5309.
 Baines, N. J., Baird, J. B. & Elmore, D. T. (1964). *Biochem. J.* **90**, 470.
 Baird, J. B., Curragh, E. F. & Elmore, D. T. (1965). *Biochem. J.* **96**, 733.
 Barrass, B. C. & Elmore, D. T. (1957a). *J. chem. Soc.* p. 3134.
 Barrass, B. C. & Elmore, D. T. (1957b). *J. chem. Soc.* p. 4830.
 Bechet, J. J. (1964). *Bull. Soc. Chim. biol., Paris*, **46**, 170.
 Bechet, J. J. & Yon, J. (1964). *Biochim. biophys. Acta*, **89**, 117.
 Bloch, K. & Clarke, H. T. (1938). *J. biol. Chem.* **125**, 275.
 Bioch. 1967, 102

- Curragh, E. F. & Elmore, D. T. (1964). *Biochem. J.* **93**, 163.
- Dadic, M., Fles, D. & Markovac, A. (1961). *Croat. Chem. Acta*, **33**, 73.
- Elmore, D. T., Baird, J. B., Roberts, D. V. & Smyth, J. J. (1964). *Abstr. 1st Meet. Fed. Europ. biochem. Soc.* no. A64.
- Elmore, D. T., Kingston, A. E. & Shields, D. B. (1963). *J. chem. Soc.* p. 2070.
- Gutfreund, H. (1955). *Trans. Faraday Soc.* **51**, 441.
- Hermann, P. & Gründig, C. A. (1963). In *Peptides*, p. 171. Ed. by Young, G. T. Oxford: Pergamon Press Ltd.
- Izumiya, N., Okazaki, H., Matsumoto, I. & Takiguchi, H. (1959). *J. Biochem., Tokyo*, **46**, 1347.
- Koshland, D. E. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 98.
- Lindley, H. (1956). *Nature, Lond.*, **178**, 647.
- Lindley, H. (1959). *Aust. J. Chem.* **12**, 296.
- Raftery, M. A. & Cole, R. D. (1963). *Biochem. biophys. Res. Commun.* **10**, 467.
- Richter, M. M. (1916). *Ber. chem. deutsch. Ges.* **49**, 2339.
- Schwert, G. W. & Eisenberg, M. A. (1949). *J. biol. Chem.* **179**, 665.
- Tesser, G. I., Nivard, R. J. F. & Gruber, M. (1964). *Biochim. biophys. Acta*, **89**, 303.
- Trowbridge, C. G., Krehbiel, A. & Laskowski, M. (1963). *Biochemistry*, **2**, 843.