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The Kinetics of Hydrolysis of Derivatives of Arginine, Homoarginine and Ornithine by Trypsin

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Trypsin catalyses the hydrolysis of suitable derivatives of basic amino acids including peptides, amides and esters. Esters are the most sensitive substrates, and it was decided to use them in the present study, which had as one of its aims the elucidation of the relationship between the length of the side chain and susceptibility to hydrolysis by trypsin. Previous investigations had shown that poly-L-ornithine and di- and tri-ornithine (Brand, Plentl & Erlanger, 1949; Erlanger, 1957) are resistant to trypsin, although α-*N*-benzoyl-L-ornithine amide is a substrate (Izumiya, Okazaki, Matsumoto & Takiguchi, 1959). The ethyl ester of α-*N*-benzoyl-DL-homoarginine (Kitagawa & Izumiya, 1959), but not the corresponding L-amide (Shields, Hill & Smith, 1959), is hydrolysed by trypsin. We decided to examine members of two series of compounds, namely (I) and (II), as potential substrates; α-*N*-toluene-*p*-sulphonyl derivatives were chosen, since α-*N*-toluene-*p*-sulphonyl-L-arginine methyl ester is a particularly sensitive substrate for trypsin. In the present paper, the kinetics of the trypsin-catalysed hydrolysis of several esters of α-*N*-toluene-*p*-sulphonyl-L-arginine and -L-homoarginine, and of the methyl ester of α-*N*-toluene-*p*-sulphonyl-L-ornithine, are described. A preliminary account of some of this work has appeared (Elmore & Baines, 1960).

EXPERIMENTAL

Materials

Trypsin (1 g. of a twice-crystallized salt-free preparation from Worthington Biochemicals Corp., Freehold, N.J., U.S.A.) was suspended in 1 mN-HCl (20 ml.), dialysed against 1 mN-HCl until the enzyme dissolved and the solution freeze-dried. The hydrochlorides of the methyl esters of α-*N*-toluene-*p*-sulphonyl-L-ornithine and -DL-ornithine (I; R = CH_3, n = 3) and of γ-amino-L-α-toluene-*p*-sulphonamidobutyric acid (I; R = CH_3, n = 2) were synthesized as described by Barrass & Elmore (1957). *N*-Benzoyl-L-arginine ethyl ester hydrochloride was prepared essentially by the method of Bergmann, Fruton & Pollok (1939) and purified by the method of Smith & Parker (1958); it had m.p. 132·5–134·5°. α-*N*-Toluene-*p*-sulphonyl-L-arginine methyl ester hydrochloride (II; R = CH_3, n = 3) was prepared by the method of Bergmann *et al.* (1939); it had m.p. 147·5–148·0°, $[\alpha]_D^{18}$ −14·4±0·3° (*c* 4 in water). Contrary to Schwert, Neurath, Kaufman & Snoke (1948) and in agreement with Ronwin (1957), we found that this ester was slowly hydrolysed above about pH 9. A good yield of α-*N*-toluene-*p*-sulphonyl-L-arginine trihydrate was isolated from a solution of the ester (II; R = CH_3, n = 3) that had been kept at pH 9·6 and room temperature for 4 days. α-*N*-Toluene-*p*-sulphonyl-L-arginine *n*-propyl ester hydrochloride

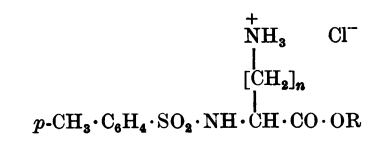
(II; R = $CH_3 \cdot CH_2 \cdot CH_2$; n = 3)

was prepared by a method worked out by E. F. Curragh & D. T. Elmore (unpublished work). Syntheses of the remaining substrates are described below.

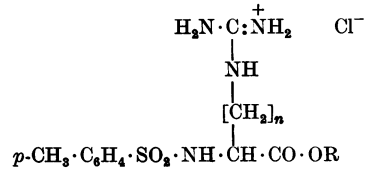
α-N-*Toluene*-p-*sulphonyl*-L-*arginine cyclohexyl ester hydrochloride.* This *compound* (II; R = C_6H_{11}, n = 3) was prepared as follows. α-*N*-Toluene-*p*-sulphonyl-L-arginine trihydrate was treated with cyclohexanol and anhydrous HCl without cooling. After 3 days the solution was concentrated under reduced pressure, and the product (83%) was crystallized from methanol–ether, when it had m.p. 165–167°, $[\alpha]_D^{17}$ + 11·7±0·3° (*c* 4 in methanol) (Found: C, 51·6; H, 6·9; N, 13·0. $C_{19}H_{31}ClN_4O_4S$ requires C, 51·1; H, 7·0; N, 12·5%).

α-N-*Toluene*-p-*sulphonyl*-L-*arginine ethyl ester hydrochloride.* This *compound* (II; R = C_2H_5, n = 3) was prepared in almost quantitative yield in a similar manner to that used for the cyclohexyl ester; crystallized from ethanol–ether, it had m.p. 113–115°, $[\alpha]_D^{18}$ −12·7±0·1° (*c* 4 in water) (Found: C, 46·1; H, 6·4; N, 14·5. $C_{15}H_{25}ClN_4O_4S$ requires C, 45·9; H, 6·4; N, 14·3%).

α-N-*Benzylidene*-L-*homoarginine.* Copper carbonate (7·0 g.) was added to a solution of L-lysine monohydrochloride (18·3 g.) in water (100 ml.). The mixture was boiled, filtered at room temperature, cooled to 0° and



(I)



(II)

brought to pH 10·7 by the addition with stirring of powdered barium hydroxide. *O*-Methylisourea sulphate (18·5 g.) was added in small portions during 3 hr., and the pH was kept at 10·7 by the addition of saturated barium hydroxide with the use of a pH-stat. The reaction mixture was stirred overnight at room temperature, adjusted to pH 6 by the addition of 50% (v/v) H_2SO_4, then boiled and filtered through Hyflo Super-Cel (Johns-Manville). The precipitate was washed several times by resuspension in boiling water, and the combined filtrate and washings were evaporated to dryness under reduced pressure. The residual solid was triturated with boiling methanol (3 × 150 ml.) to remove a small quantity of a colourless substance having m.p. 274–276° (Found: C, 51·8; H, 8·4; N, 22·3. $C_8H_{15}N_3O_2$ requires C, 51·9; H, 8·2; N, 22·7%). This by-product was not further examined. The copper complex of homoarginine was dissolved in water (250 ml.) and the solution was slightly acidified, heated, treated with H_2S for 1 hr. and filtered through Hyflo Super-Cel. The filtrate was brought to pH 11·5 with saturated barium hydroxide and shaken overnight with a solution of benzaldehyde (15 ml.) in ether (150 ml.). α-N-*Benzylidene*-L-*homoarginine* (21·0 g.) was collected, washed with ice–water and then with ether, and dried, when it had m.p. 212–213° (Found: C, 60·2; H, 7·1; N, 19·9. $C_{14}H_{20}N_4O_2$ requires C, 60·2; H, 7·3; N, 20·3%).

α-N-*Toluene*-p-*sulphonyl*-L-*homoarginine*. This compound (II; R = H, $n = 4$) was prepared as follows.

(*a*) A solution of α-*N*-benzylidene-L-homoarginine (10·0 g.) in N-HCl (50 ml.) was heated to boiling, allowed to cool during 1 hr. and then extracted with ether. The solution was adjusted to pH 8·5–9·0 with 2N-NaOH and stirred overnight with a solution of toluene-*p*-sulphonyl chloride (7·25 g.) in ether with periodic additions of alkali to maintain the pH in the above range. α-N-*Toluene*-p-*sulphonyl*-L-*homoarginine* (8·9 g.) was collected, washed with ether and recrystallized from hot water, when it had m.p. 200–202° after softening from 180°, $[\alpha]_D^{18} - 13{\cdot}8 \pm 0{\cdot}3°$ (*c* 4 in N-HCl) (Found: C, 48·9; H, 6·2; N, 16·5. $C_{14}H_{22}N_4O_4S$ requires C, 49·1; H, 6·5; N, 16·4%).

(*b*) α-*N*-Toluene-*p*-sulphonyl-L-lysine (3·0 g.) (Barrass & Elmore, 1957) in 2N-NaOH (11 ml.) was allowed to react with *S*-methylisothiouronium iodide (2·4 g.) at room temperature. The pH was kept at about 10·5, and the course of the reaction was followed by periodically testing a drop of solution with ninhydrin. After 2 days, the solution was brought to pH 12, extracted several times with ethyl acetate and then adjusted to pH 7 with 2N-HCl. The resulting α-*N*-toluene-*p*-sulphonyl-L-homoarginine (1·33 g.) was recrystallized from water, when it had m.p. 197–198° after softening from 180°. Specimens obtained by the two methods had identical infrared-absorption spectra.

α-N-*Toluene*-p-*sulphonyl*-L-*homoarginine methyl ester hydrochloride*. This compound (II; R = CH_3, $n = 4$) was prepared as follows. α-*N*-Toluene-*p*-sulphonyl-L-homoarginine (5·0 g.) was esterified with methanol saturated with anhydrous HCl. Evaporation yielded the *ester hydrochloride* (5·0 g.), which had m.p. 140–141° after recrystallization from methanol–ether, $[\alpha]_D^{18} - 13{\cdot}8 \pm 0{\cdot}4°$ (*c* 4 in water) (Found: C, 45·8; H, 6·3; N, 14·4. $C_{15}H_{25}ClN_4O_4S$ requires C, 45·9; H, 6·4; N, 14·3%).

α-N-*Toluene*-p-*sulphonyl*-L-*homoarginine ethyl ester hydrochloride*. This compound (II; R = C_2H_5, $n = 4$) was prepared as follows. Esterification of α-*N*-toluene-*p*-sulphonyl-L-homoarginine (3·0 g.) was effected with ethanol saturated with anhydrous HCl. Since the hydrochloride could not at first be crystallized, ether was added until the ethanolic solution of hydrochloride was faintly turbid, then lithium hydroxide solution was added dropwise to obtain maximum precipitation of the free *ester*. The latter (2·0 g.), which is presumably zwitterionic, was filtered off, washed with ice–water and then with ether, and dried, when it had m.p. 189–191° (Found: C, 51·1; H, 6·6. $C_{16}H_{26}N_4O_4S$ requires C, 51·9; H, 7·1%). The free ester was suspended in ethanol and dissolved by passage of anhydrous HCl. The addition of ethyl acetate and ether followed by storage at 0° caused the hydrochloride (1·2 g.) to crystallize. After recrystallization from ethanol–ethyl acetate–ether, it had m.p. 92–94°, $[\alpha]_D^{18} - 12{\cdot}0 \pm 0{\cdot}3°$ (*c* 4 in water) (Found: C, 47·0; H, 6·5; N, 14·0) $C_{16}H_{27}ClN_4O_4S$ requires C, 47·2; H, 6·7; N, 13·8%).

α-N-*Toluene*-p-*sulphonyl*-L-*homoarginine* n-*propyl ester hydrochloride*. The preparation of this *compound* (II; R = $CH_3 \cdot CH_2 \cdot CH_2$, $n = 4$) (2·15 g.) from α-*N*-toluene-*p*-sulphonyl-L-homoarginine (2·0 g.) was similar to that of the foregoing compound. Recrystallized from propan-1-ol–ether, it had m.p. 104–107° after softening from 100°, $[\alpha]_D^{18} - 10{\cdot}1 \pm 0{\cdot}1°$ (*c* 4 in water) (Found: C, 48·3; H, 6·9; N, 13·2. $C_{17}H_{29}ClN_4O_4S$ requires C, 48·5; H, 6·9; N, 13·3%).

α-N-*Toluene*-p-*sulphonyl*-L-*homoarginine cyclohexyl ester*. The free *ester* (II; R = C_6H_{11}, $n = 4$) (1·4 g.) was prepared from α-*N*-toluene-*p*-sulphonyl-L-homoarginine (2·0 g.) by the method described for the corresponding ethyl ester; it had m.p. 203–205° (decomp.) $[\alpha]_D^{17} + 12{\cdot}6 \pm 0{\cdot}3°$ (*c* 4 in methanol containing 1 equiv. of HCl) (Found: C, 56·2; H, 7·4; N, 13·5. $C_{20}H_{30}N_4O_4S$ requires C, 56·6: H, 7·6; N, 13·2%). The hydrochloride could not be obtained in crystalline form.

Kinetic technique

The progress of ester hydrolysis was followed on a pH-stat consisting of a Radiometer (Copenhagen) autotitrator TTT 1a and a pen-recorder. The latter recorded the volume of alkali added from a micrometer syringe as a function of time. The drive from a continuously running electric motor was applied to the micrometer syringe and the lead-screw, which carried the pen, through a gear train and electromagnetic clutch. The latter, which eliminated 'hunting' or over-running, was activated by the magnetic relay of the autotitrator when the pH differed from the set value. Combined glass and calomel electrodes (Radiometer GK 2021 B) were used, and were standardized with 25 mM-phosphate (pH 6·86 at 25°) (Bates, 1954) and 50 mM-potassium hydrogen phthalate buffers. The buffer setting seldom varied by more than 0·02 pH unit from day to day. Precision within the range pH 6·0–8·0 was improved by using the Radiometer external meter (PHA 622). Plug-in or immersion-type Radiometer temperature compensators were used. The proportional band-width was set as low as possible (usually 0·10–0·15 pH unit), with care being taken to minimize relay 'chatter'. All kinetic runs were carried out with efficient stirring in a thermostat controlled to ±0·01° under a stream of nitrogen, which was freed from CO_2 and saturated with water vapour at the temperature of the experiment. The reaction vessels were of two types; the smaller (about 15 ml.) was used for runs when the initial substrate concentration, $[S]_0$, was not less than 0·5 mM, and the larger (about 150 ml.) was used when $[S]_0$ was less than 0·5 mM. The larger vessel

was fitted with a Perspex lid. Carbonate-free NaOH, stored in waxed bottles fitted with soda-lime guard-tubes, was used for all titrations. A solution of trypsin in 1 mN-HCl ($E^{1\,cm.}_{280\,m\mu}$ not less than 0·2) was prepared daily and volumes of 10–120 μl. were dispensed from a micrometer syringe. In the upper part of this range or with very dilute NaOH, the theoretical amount of alkali was added to the substrate solution before the addition of trypsin in order to neutralize the acid present in the enzyme solution. With α-*N*-toluene-*p*-sulphonyl-L-ornithine methyl ester, however, excess of alkali would have caused lactamization (Curragh & Elmore, 1962), and trypsin was dissolved, therefore, in 0·1 mN-HCl. Trypsin concentrations were determined by measurement of the extinction at 280 mμ. In agreement with Green (1953), we found that a solution with $E^{1\,cm.}_{280\,m\mu}$ 1·0 contained 0·640 mg. of protein/ml. The mol.wt. of trypsin was taken to be 23800 (Cunningham, 1954). Unless otherwise stated, experiments were carried out in a solution which was 0·1 M with respect to sodium chloride and 2 mM with respect to calcium chloride (Green & Neurath, 1953).

In early experiments, in which more dilute solutions of trypsin were dispensed from glass pipettes of 1–3 ml. capacity, the results showed considerable dispersion. Since chymotrypsin (Bixler & Niemann, 1959) and ribonuclease (Shapira, 1959) are strongly adsorbed on glass surfaces, we carried out some experiments to ascertain if trypsin behaves in a similar manner. Samples of the same solution of trypsin were shaken gently with glass powder at pH 3 or pH 8 at 20°, the glass powder was removed by centrifuging, and the hydrolytic activity of the supernatant was determined with α-*N*-toluene-*p*-sulphonyl-L-arginine methyl ester as substrate at 25° and pH 8·6. These experiments showed that trypsin, at a concentration of 5 μg./ml. or less, is strongly adsorbed by soda-glass at pH 8; at pH 3, adsorption was considerably less important than at pH 8. When small volumes of concentrated trypsin solutions were dispensed from a micrometer syringe, such errors, as judged by the dispersion of the kinetic results, were not important.

Method of computation

Throughout our work, velocities of reaction and values for $K_{m(app.)}$ and kinetic constants have been computed on the English Electric DEUCE digital computer by methods described by Elmore, Kingston & Shields (1963).

The mechanism of action of trypsin and chymotrypsin can be summarized as shown in Scheme 1: the two products of the reaction are formed in separate steps of the process.

The kinetic equations governing the reaction mechanism outlined in Scheme 1 have been derived by Gutfreund & Sturtevant (1956*a*, *b*) for the pre-steady and steady states. The apparent zero-order rate constant, $k'_{3(app.)}$, at any pH value, is given by the equation:

$$1/k'_{3(app.)} = 1/k'_2 + 1/k'_3 \qquad (1)$$

where

$$k'_2 = k_2K_1/(K_1 + [H^+]) \qquad (2)$$

and

$$k'_3 = k_3K_2/(K_2 + [H^+]) \qquad (3)$$

The apparent Michaelis constant, $K_{m(app.)}$, is related to the true Michaelis constant by the equation:

$$K_{m(app.)} = \frac{k'_3}{k'_2 + k'_3} \cdot K_m = \frac{k'_3(k_{-1} + k'_2)}{k_1(k'_2 + k'_3)} \qquad (4)$$

Determination of the apparent zero-order rate constant, $k'_{3(app.)}$, over a range of pH values below the optimum pH value for the reaction leads to an apparent dissociation constant, $K_{(app.)}$, for the ionizing group, which is defined by the equation:

$$k'_{3(app.)} = k_{3(app.)}K_{(app.)}/(K_{(app.)} + [H^+]) \qquad (5)$$

where:

$$k_{3(app.)} = 1/k_2 + 1/k_3 \qquad (6)$$

From eqns. (1), (2), (3) and (5), it can be shown that:

$$K_{(app.)} = \frac{K_1K_2(k_2 + k_3)}{k_2K_1 + k_3K_2} \qquad (7)$$

It follows that if $k_2 \gg k_3$ and $K_2 \not\gg K_1$ (i.e. deacylation is rate-determining):

$$K_{(app.)} \sim K_2$$

On the other hand, if $k_2 \ll k_3$ and $K_2 \not\ll K_1$ (i.e. acylation is rate-determining):

$$K_{(app.)} \sim K_1$$

In general, if $k_2 = nk_3$ and $K_1 = mK_2$:

$$K_{(app.)} = \frac{K_1(n+1)}{mn+1} = \frac{mK_2(n+1)}{mn+1} \qquad (8)$$

If

$m > 1$, then $K_1 > K_{(app.)} > K_2$, and if

$m < 1$, then $K_1 < K_{(app.)} < K_2$

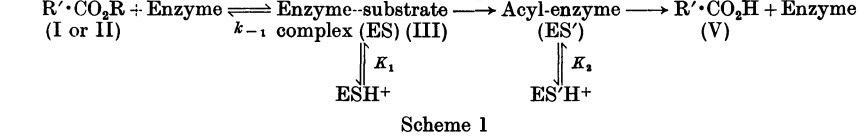
Below the pH optimum, the relation between k'_3 and $[H^+]$ is given by the equation:

$$1/k'_{3(app.)} = 1/k_{3(app.)} + [H^+]/k_3K_{(app.)} \qquad (9)$$

The required regression line was computed by the method of weighted least squares. The worst point was tested for significance by using essentially the same method as that employed for the calculation of kinetic constants (Elmore *et al.* 1963).

RESULTS AND DISCUSSION

The results of our investigation are summarized in Tables 1–3, but, before discussing them in detail, it is necessary to mention some salient aspects of the subject.



Scheme 1

Several esters of α-*N*-benzoyl-L-arginine, at concentrations high enough to ensure that zero-order kinetics were obeyed, were found to be hydrolysed by trypsin at the same rate (Schwert & Eisenberg, 1949). The inference to be drawn from these experiments is that a common intermediate, (α-*N*-benzoyl-L-arginyl)trypsin, is formed and that the deacylation of this is rate-determining. The hydroxyl group in the serine residue at the active centre is probably the site of acylation. Strong evidence for such intermediates has been obtained with chymotrypsin-catalysed reactions, and has been reviewed by Desnuelle (1960) and Bender (1960). The hydrolysis of *N-trans*-cinnamoylimidazole is catalysed by either trypsin or chymotrypsin, and the formation of an intermediate cinnamoyl derivative of the enzyme has been demonstrated in each case (Bender, Schonbaum & Zerner, 1962; Bender & Kaiser, 1962). Both the enzyme–substrate complex and the acyl-enzyme are assumed to contain basic groups that are essential for the reaction to proceed. The pK values of the conjugate acids, ESH^+ and $ES'H^+$, are not necessarily identical. Indeed, if an imidazole group is implicated (Bender, 1960), hydrogen-bonding between the hydroxyl group of the serine residue and imidazole could occur in the enzyme–substrate complex but not in the acyl-enzyme. On the other hand, hydrogen-bonding could occur between the imidazolium group and the carbonyl

Table 1. $K_{m(app.)}$ *values at* pH 8·4 *and* 25° *for the trypsin-catalysed hydrolysis of derivatives of arginine and homoarginine*

Results are given as means±S.D.

Substrate	$10^6 K_{m(app.)}$ (M)
(II; R = CH_3, n = 3)	6·4±1·2
(II; R = $CH_3{\cdot}CH_2{\cdot}CH_2$, n = 3)	6·6±0·8
(II; R = C_6H_{11}, n = 3)	8·2±0·9
(II; R = CH_3, n = 4)	332±2
(II; R = C_2H_5, n = 4)	289±12
(II; R = $CH_3{\cdot}CH_2{\cdot}CH_2$, n = 4)	257±10
(II; R = C_6H_{11}, n = 4)	238±3

Table 2. $k_{3(app.)}$ *values at* pH 8·4 *for the trypsin-catalysed hydrolysis of derivatives of arginine and homoarginine*

Results are given as means±S.D. with the numbers of determinations in parentheses. Values marked with an asterisk were obtained from determinations of $K_{m(app.)}$.

	$k_{3(app.)}$ (sec.$^{-1}$)				
Temperature	15°	20°	25°	30°	37°
Substrate:					
(II; R = CH_3, n = 3)	43·62 ±7·74 (3)	54·18 ±5·21 (4)	74·99 ±1·72 (4)	94·38 ±6·79 (4)	149·75 ±8·55 (4)
(II; R = C_2H_5, n = 3)	37·61 ±4·82 (3)	50·98 ±2·73 (3)	74·10 ±2·03 (4)	89·97 ±11·46 (4)	142·40 ±4·91 (4)
(II; R = $CH_3{\cdot}CH_2{\cdot}CH_2$, n = 3)	37·72 ±2·89 (2)	54·37 ±1·56 (2)	76·58 ±1·33 (4)	94·48 ±11·05 (4)	150·73 ±6·40 (5)
(II; R = C_6H_{11}, n = 3)	43·17 ±4·94 (3)	55·30 ±6·84 (3)	76·85 ±3·69 (4)	96·23 ±5·66 (4)	158·28 ±5·62 (4)
α-Benzoyl-L-arginine methyl ester	7·086 ±0·174 (4)	10·48 ±0·66 (4)	14·38 ±0·62 (4)	21·49 ±1·38 (6)	—
(II; R = CH_3, n = 4)	2·286 ±0·098 (7)	3·042 ±0·108 (8)	4·041 ±0·212 (8)	5·331 ±0·231 (8)	—
(II; R = C_2H_5, n = 4)	—	—	4·077 ±0·058* (2)	—	—
(II; R = $CH_3{\cdot}CH_2{\cdot}CH_2$, n = 4)	—	—	4·263 ±0·037* (2)	—	—
(II; R = C_6H_{11}, n = 4)	—	—	3·461 ±0·014* (2)	—	—

Table 3. *Activation constants for the trypsin-catalysed hydrolysis of derivatives of arginine and homoarginine*

Results are given as means±S.D.

Substrate	Energy of activation (E_a) (kcal./mole)	Enthalpy of activation ($\Delta H^\ddagger$) (kcal./mole at 25°)	Entropy of activation ($\Delta S^\ddagger$) (e.u. at 25°)
(II; R = CH_3, n = 3)	10·37±0·45	9·77±0·45	−17·2±1·5
(II; R = C_2H_5, n = 3)	10·50±0·45	9·91±0·45	−16·8±1·5
(II; R = $CH_3{\cdot}CH_2{\cdot}CH_2$, n = 3)	10·94±0·42	10·35±0·42	−15·2±1·4
(II; R = C_6H_{11}, n = 3)	10·91±0·57	10·31±0·57	−15·3±1·9
α-Benzoyl-L-arginine methyl ester	12·51±0·35	11·92±0·35	−13·2±1·2
(II; R = CH_3, n = 4)	9·81±0·07	9·22±0·07	−24·8±0·2

group in the acyl-enzyme. Consequently, the pK of the ionizing group in the enzyme–substrate complex would be expected to be lower than that of the group in the acyl-enzyme. Such a difference has been found in the chymotrypsin-catalysed hydrolysis of p-nitrophenyl acetate (Gutfreund & Sturtevant, 1956*a*).

Kinetics of hydrolysis of derivatives of arginine. At low substrate concentrations, the trypsin-catalysed hydrolysis of the methyl, n-propyl and cyclohexyl esters of α-N-toluene-p-sulphonyl-L-arginine were found to obey Michaelis–Menten kinetics, and the values of $K_{m(\text{app.})}$ are given in Table 1. Owing to the low magnitude of $K_{m(\text{app.})}$ it was possible to measure initial velocities for only a restricted range of substrate concentrations. The values of $K_{m(\text{app.})}$ were statistically indistinguishable for the three esters. In agreement with Trowbridge & Laskowski (1962), we found that the foregoing reactions departed from Michaelis–Menten kinetics at high substrate concentrations. Trowbridge & Laskowski (1962) suggest that activation by the substrate occurs in the trypsin-catalysed hydrolysis of α-N-toluene-p-sulphonyl-L-arginine methyl ester, and they have computed the dissociation constants ($1{\cdot}2 \times 10^{-5}$M and $6{\cdot}5 \times 10^{-2}$M) of the complexes containing one and two molecules of substrate respectively as well as the apparent first-order rate constants (60 sec.$^{-1}$ and 440 sec.$^{-1}$ respectively) for their decomposition. Previous work indicated a much higher value for $K_{m(\text{app.})}$ for the methyl ester. Thus Schwert *et al.* (1948) recorded that the kinetics of its trypsin-catalysed hydrolysis approached first-order in the pH range 9–10. Martin, Golubow & Axelrod (1959) report $K_m = 4{\cdot}9 \times 10^{-3}$M at pH 8·0 and 25° in the presence of 0·1M-calcium chloride, Lorand & Rule (1961) report $K_m = 3{\cdot}6 \times 10^{-3}$M at pH 7·7 and 30°, and the results of Ronwin (1959), which are in graphical form, indicate that $K_m \sim 6 \times 10^{-3}$M over the pH range 7·8–8·6 at 38° in the presence of 6·6 mM-calcium chloride. Presumably, these values were measured under conditions when activation by the substrate occurred.

The value of $k_{3(\text{app.})}$ has been determined for the methyl, ethyl, n-propyl and cyclohexyl esters of α-N-toluene-p-sulphonyl-L-arginine (II; R = CH_3, C_2H_5, $CH_3{\cdot}CH_2{\cdot}CH_2$ and C_6H_{11} respectively, $n = 3$) at five temperatures: the value for the methyl ester at 25° is in fair agreement with that obtained by Trowbridge & Laskowski (1962). A feature of our results is that $k_{3(\text{app.})}$ is not significantly different for the four esters. It may be concluded that the acylation of enzyme, i.e. (III)→(IV), is fast, and the rate-determining step in each case is the deacylation of (α-N-toluene-p-sulphonyl-L-arginyl)trypsin, i.e. (IV)→(V). For these substrates, therefore, $k'_2 \gg k'_3$. Moreover, the energies, enthalpies and entropies of activation (Table 3) did not differ significantly from one substrate to another. Martin *et al.* (1959) found that $k_{3(\text{app.})}$ was 187 sec.$^{-1}$ at pH 8 and 25°, whereas the results of Hummel (1959) lead to a value of 106 sec.$^{-1}$ at pH 8·1 and 30° in 10 mM-calcium chloride. These values are considerably higher than ours or those of Trowbridge & Laskowski (1962), and were presumably determined under conditions where activation by the substrate obtained.

The effect of pH on $k'_{3(\text{app.})}$ has been studied for the trypsin-catalysed hydrolysis of the methyl ester (II; R = CH_3, $n = 3$). The substrate concentration used for these experiments (0·2 mM) was adequate to ensure that maximum velocity was reached for the normal reaction, but not high enough to cause significant activation by the substrate. Computation of the regression line of $1/v$ on $[H^+]$ indicated that a group of p$K_{(\text{app.})}$ $7{\cdot}85 \pm 0{\cdot}05$ required to be dissociated for deacylation of the acylated enzyme to proceed. The p$K_{(\text{app.})}$ is higher than expected for an imidazole group, which is believed to participate in trypsin catalysis, and is higher than that found to be involved in the trypsin-catalysed hydrolysis of α-N-benzoyl-L-arginine ethyl ester (Gutfreund, 1955). No satisfactory explanation is available for this observation at present, although the acid strength of an imidazolium group is rather sensitive to its molecular environment (Barnard & Stein, 1958). Alternatively, although $k_2 \gg k_3$, we have no definite information about the relative magnitudes of K_1 and K_2 and, if for some reason $K_2 \gg K_1$, k_3K_2 may not be negligible compared with k_2K_1 (eqn. 7), in which case

$$K_{(\text{app.})} < K_2 \text{ (i.e. } pK_{(\text{app.})} > pK_2)$$

(Bender, Clement, Kézdy & Zerner, 1963).

Schwert & Eisenberg (1949) found that the methyl, ethyl, isopropyl, cyclohexyl and benzyl esters of α-N-benzoyl-L-arginine were hydrolysed by trypsin at the same rate. Again it appears that $k_2 \gg k_3$ and that the rate-determining step is the deacylation of the acylated enzyme, (α-N-benzoyl-L-arginyl)trypsin. These workers also studied the effect of temperature on the rate of trypsin-catalysed hydrolysis of the methyl ester and they obtained values of 10·6 kcal./mole, −16·5 e.u. and 26·7 sec.$^{-1}$ respectively for $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $k_{3(\text{app.})}$. The last two values, however, were computed on the assumption that trypsin has a mol.wt. of 36000. Recalculation, by using a value of 23800 for the mol.wt. of trypsin, gives −12·5 e.u. and 17·65 sec.$^{-1}$ for $\Delta S^{\ddagger}$ and $k_{3(\text{app.})}$ at 25° respectively. By using the corresponding ethyl ester in 30 mM-calcium chloride, Terminiello, Sri Ram, Bier & Nord (1955) obtained values of 12·7 kcal./mole, −10·7 e.u. and 14·29 sec.$^{-1}$ for $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $k_{3(\text{app.})}$ at 25°

respectively. We have determined these parameters at pH 8·4 in 1 mM-calcium chloride, and the results are in reasonable agreement with those of Terminiello *et al.* (1955). The rates of the trypsin-catalysed hydrolysis of α-*N*-toluene-*p*-sulphonyl-L-arginine methyl ester and α-*N*-benzoyl-L-arginine ethyl ester are independent of ionic strength over a wide range.

Kinetics of hydrolysis of derivatives of homoarginine. The methyl, ethyl, *n*-propyl and cyclohexyl esters of α-*N*-toluene-*p*-sulphonyl-L-homoarginine (II; R = CH_3, C_2H_5, $CH_3 \cdot CH_2 \cdot CH_2$ and C_6H_{11} respectively, $n = 4$) have been examined as substrates for trypsin. For all of these compounds, $K_{m(app.)}$ was very much higher than for esters of α-*N*-toluene-*p*-sulphonyl-L-arginine (Table 1). Although there is no evidence that $K_{m(app.)}$ is purely an equilibrium constant, it is likely that the homoarginine derivatives are less firmly bound than the arginine compounds to the enzyme. There was a small but definite tendency for the $K_{m(app.)}$ to decrease with increasing size of the alkoxy group of the ester (Table 1). This behaviour contrasts with that of the arginine derivatives, and it is possible that the homoarginine substrates alter the conformation of the enzyme sufficiently for an additional hydrophobic group to be implicated in the binding of substrate. Alternatively, it is possible that such a group is operative with both types of substrate, but that, with arginine derivatives, the guanidino group, for example, makes an overwhelming contribution to the binding of the whole molecule.

For the hydrolyses of the methyl, ethyl and *n*-propyl esters of α-*N*-toluene-*p*-sulphonyl-L-homoarginine, $k_{3(app.)}$ was statistically indistinguishable at 25° (Table 2), and again it may be concluded that $k_2 \gg k_3$ and that deacylation of the acylated enzyme, i.e. (IV)→(V), is rate-determining. Since $k_{3(app.)}$ for the bulkier cyclohexyl ester (II; R = C_6H_{11}, $n = 4$) is somewhat lower, it appears that the acylation step, i.e. (III)→(IV), is partly rate-determining for the hydrolysis of this ester. The value of $k_{3(app.)}$ has been determined at four temperatures for α-*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester, and the activation constants have been computed (Table 3). The energies and enthalpies of activation for the hydrolysis of the methyl esters of α-*N*-toluene-*p*-sulphonyl-L-arginine and L-homoarginine are not significantly different. There is, however, a distinct difference between the entropies of activation: $\Delta S^{\ddagger}$ for the hydrolysis of the homoarginine derivative is much more negative than it is for the hydrolysis of the arginine substrate. For the hydrolysis of both substrates, the activation constants refer to the deacylation step, i.e. (IV)→(V), and it is possible that the highly negative value of $\Delta S^{\ddagger}$ for α-*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester reflects an increased binding of solvent, an increased distortion of the molecular conformation, or a more complex distribution of charges in the transition state.

Determination of $k'_{3(app.)}$ has been carried out over a range of pH for the trypsin-catalysed hydrolysis of the methyl ester (II; R = CH_3, $n = 4$), and the $pK_{(app.)}$ was found to be $6{\cdot}93 \pm 0{\cdot}04$. This is distinctly lower than the $pK_{(app.)}$ for the hydrolysis of the corresponding ester in the arginine series, but there is as yet no satisfactory explanation for this difference. The $pK_{(app.)}$ for the hydrolysis of the homoarginine derivative, however, is within the expected range for the dissociation of an imidazolium group.

Kinetics of hydrolysis of α-N-*toluene*-p-*sulphonylornithine methyl ester.* The trypsin-catalysed hydrolysis of α-*N*-toluene-*p*-sulphonyl-L-ornithine methyl ester (I; R = CH_3, $n = 3$) could not be studied satisfactorily at pH 8·4 owing to the concomitant lactamization of the substrate, a reaction which is catalysed by OH^- ions (Curragh & Elmore, 1962). A limited examination of the trypsin-catalysed hydrolysis of this substrate was made, therefore, at pH 7·0, when lactamization was insignificant. The value of $K_{m(app.)}$ was $1{\cdot}53 \pm 0{\cdot}08 \times 10^{-2}$ M at 25° and was therefore much higher than $K_{m(app.)}$ for any of the other trypsin substrates considered above. The value of $k'_{3(app.)}$, $3{\cdot}34 \pm 0{\cdot}09$ sec.$^{-1}$, however, was somewhat higher than for α-*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester under similar conditions of temperature and pH. The value of $k_{3(app.)}$ and the relative magnitudes of k_2 and k_3 are unknown for the ornithine derivative, and detailed comparison with other substrates of trypsin is not possible.

The trypsin-catalysed hydrolysis of α-*N*-toluene-*p*-sulphonylornithine methyl ester appears not to be completely stereospecific. With the DL-ester, it was found that the rate of alkali uptake in the presence of trypsin was greater than when the L-ester of half the molar concentration was used as substrate. Moreover, when the DL-ester was hydrolysed by trypsin, alkali uptake continued beyond the value representing 50% hydrolysis. This was shown to be not due to lactamization in the following way: after hydrolysis had proceeded to the point when the alkali uptake was 74·8% of the theoretical value required for complete hydrolysis, a Sørensen titration indicated that only 8·6% of the total amount of ester had lactamized. A control experiment without enzyme showed that the alkali uptake did not result from chemical hydrolysis.

The lower homologue of α-*N*-toluene-*p*-sulphonyl-L-ornithine methyl ester, methyl γ-amino-L-α-toluene-*p*-sulphonamidobutyrate, is not signifi-

cantly hydrolysed by trypsin at pH 7·0. Izumiya *et al.* (1959) were unable to detect any hydrolysis of γ-amino-L-α-benzamidobutyramide by trypsin.

SUMMARY

1. The synthesis of several new substrates for trypsin is described.

2. The occurrence of activation by the substrate during the trypsin-catalysed hydrolysis of α-*N*-toluene-*p*-sulphonyl-L-arginine methyl ester has been confirmed. The kinetics of the hydrolysis of this and the corresponding ethyl, *n*-propyl and cyclohexyl esters are statistically indistinguishable. It is concluded that $k_{3(app.)}$ refers to the deacylation of (α-*N*-toluene-*p*-sulphonyl-L-arginyl)-trypsin.

3. Esters of α-*N*-toluene-*p*-sulphonyl-L-homoarginine are substrates for trypsin, although $K_{m(app)}$ is considerably higher than for the corresponding arginine compounds. The identity of $k_{3(app.)}$ for the methyl, ethyl and *n*-propyl esters indicates that the deacylation of (α-*N*-toluene-*p*-sulphonyl-L-homoarginyl)trypsin is rate-determining. The difference between the values of $k_{3(app.)}$ for the arginine and homoarginine derivatives is mainly due to a difference in entropy of activation.

4. The variation of $k'_{3(app.)}$ with pH for α-*N*-toluene-*p*-sulphonyl-L-arginine methyl ester suggests that a group with $pK_{(app.)}$ $7{\cdot}85 \pm 0{\cdot}05$ must be dissociated for deacylation of the intermediate acylated enzyme to proceed. With α-*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester, the $pK_{(app.)}$ value is $6{\cdot}93 \pm 0{\cdot}04$.

5. Trypsin catalyses the hydrolysis of α-*N*-toluene-*p*-sulphonylornithine methyl ester at pH 7·0, but the reaction is not completely stereospecific.

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