Kinetics and Mechanism of Catalysis by Proteolytic Enzymes

THE KINETICS OF HYDROLYSIS OF ESTERS OF γ -GUANIDINO-L- α -TOLUENE-*p*-SULPHONAMIDOBUTYRIC ACID BY BOVINE TRYPSIN AND THROMBIN

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(Received 18 December 1964)

1. Esters of γ -guanidino-L- α -toluene-*p*-sulphonamidobutyric acid (α -*N*-toluene-*p*-sulphonyl-L-norarginine) have been synthesized and shown to be hydrolysed by bovine trypsin and thrombin. As substrates for these enzymes, they were better than esters of α -*N*-toluene-*p*-sulphonyl-L-homoarginine or of α -*N*-toluene-*p*-sulphonyl-L-ornithine but not as good as esters of α -*N*-toluene-*p*-sulphonyl-L-arginine. 2. With trypsin as catalyst, the methyl and propyl esters are hydrolysed at the same rate at high substrate concentrations and hence deacylation of the acyl-enzyme appears to be rate-determining. In the presence of thrombin, however, the methyl ester is hydrolysed much faster than the *n*-propyl ester. 3. The variation of k_0 with pH indicates that groups with $pK_{(app.)}$ values of 7.05 ± 0.02 and 6.53 ± 0.02 must be dissociated in trypsin and thrombin respectively for hydrolysis to proceed. 4. Activation constants have been determined for the trypsin-catalysed hydrolysis of methyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate and have been compared with the corresponding constants for the hydrolysis of homologous substrates. 5. Cholate increases k_0 and decreases K_m ; the effects are more pronounced with thrombin than with trypsin.

It is generally accepted that derivatives of lysine and arginine are the best substrates for bovine pancreatic trypsin and bovine thrombin. Recently, however, it has been shown that these enzymes are not absolutely specific with respect to the length of the side chain of the substrate (Baines, Baird & Elmore, 1964; Curragh & Elmore, 1964; Elmore, Baird, Roberts & Smyth, 1964). In a study of the behaviour of trypsin and thrombin towards esters of ω -amino-L- α -toluene-*p*-sulphonamido acids (I), it was found that α -*N*-toluene-*p*-sulphonyl-L-ornithine methyl ester (I; $n = 3$) was hydrolysed by both enzymes, although much more slowly than α -*N*-toluene-*p*-sulphonyl-L-lysine methyl ester (I; $n = 4$). Neither enzyme, however, effected the hydrolysis of methyl γ -amino-L- α -toluene-*p*-sulphonamidobutyrate (I; $n = 2$). In the related series (II), trypsin catalysed the hydrolysis of esters of α -*N*-toluene-*p*-sulphonyl-L-homoarginine (II; $n = 4$), although considerably less effectively than with the arginine derivatives (II; $n = 3$). Thrombin, on the other hand, did not hydrolyse the homoarginine derivatives. Indeed, α -*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester was found to be a potent inhibitor of both the esterase and clotting activities of thrombin. In the present paper, we have studied the behaviour of trypsin and thrombin towards the lower members in the series

of esters of ω -guanidino- α -toluene-*p*-sulphonamido acids (II; $n = 2, 1$).

EXPERIMENTAL

Materials

The thrombin used in this investigation was 'topical thrombin' obtained from Parke, Davis and Co. Ltd., and its activity was assayed with α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester as described by Curragh & Elmore (1964) and expressed in the units defined by Ehrenpreis &

Soheraga (1957). Trypsin, twice-crystallized, salt-free (Worthington Biochemicals Corp., Freehold, N.J., U.S.A.), was dialysed against 1M-N-HCl and freeze-dried; its activity was checked with α -N-toluene-*p*-sulphonyl-L-arginine methyl ester (Baines *et al.* 1964). γ -Guanidino-L- α -toluene-*p*-sulphonamidobutyric acid and β -guanidino-L- α -toluene-*p*-sulphonamidopropionic acid were synthesized by the method of Rudinger, Poduška & Zaoral (1960). In our experience, the latter compound melted as described by these authors but analysed for a hemihydrate rather than a dihydrate (Found: C, 43.1; H, 5.2; N, 18.1. $C_{11}H_{16}N_4O_4S \cdot \frac{1}{2}H_2O$ requires C, 42.7; H, 5.5; N, 18.1%). Cholic acid was twice recrystallized from ethanol and had m.p. 200–201°.

Methyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate hydrochloride. γ -Guanidino-L- α -toluene-*p*-sulphonamidobutyric acid (5.0 g.) was esterified with methanol saturated with dry HCl. Evaporation afforded the ester hydrochloride (II; R = CH₃, *n* = 2) (4.7 g.), which had m.p. 190–191° after recrystallization from methanol-ether, $[\alpha]_D^{25} + 19.6 \pm 0.4^\circ$ (c 4 in methanol) (Found: C, 42.4; H, 5.6; N, 14.9. $C_{13}H_{21}ClN_4O_4S$ requires C, 42.8; H, 5.8; N, 15.4%).

Propyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate hydrochloride. γ -Guanidino-L- α -toluene-*p*-sulphonamidobutyric acid (5.0 g.) was converted into the ester hydrochloride (II; R = CH₃·CH₂·CH₂, *n* = 2) (5.0 g.) by treatment with *n*-propanol saturated with dry HCl. After recrystallization from propan-1-ol-ether, it had m.p. 128–129°, $[\alpha]_D^{25} + 18.6 \pm 0.3^\circ$ (c 4 in ethanol) (Found: C, 45.8; H, 6.2; N, 14.5. $C_{15}H_{25}ClN_4O_4S$ requires C, 45.9; H, 6.4; N, 14.3%).

Methyl β -guanidino-L- α -toluene-*p*-sulphonamidopropionate hydrochloride. Esterification of β -guanidino-L- α -toluene-*p*-sulphonamidopropionic acid (5.0 g.) with methanol saturated with dry HCl gave a product that did not solidify when the solution was evaporated and could not be crystallized from rigorously dried solvents. Solution of the oil in moist methanol and the addition of ethyl acetate and ether followed by storage at 0° caused the ester hydrochloride (II; R = CH₃, *n* = 1) (4.5 g.) to crystallize as the hemihydrate. Recrystallized from ethyl acetate-ether, it had m.p. 146–148° with softening from 86° (Found: C, 39.9; H, 5.4; N, 16.0. $C_{12}H_{19}ClN_4O_4S \cdot \frac{1}{2}H_2O$ requires C, 40.0; H, 5.6; N, 15.6%).

Behaviour of methyl β -guanidino-L- α -toluene-*p*-sulphonamidopropionate under basic conditions. The ester, when kept in aqueous solution at constant pH in the range pH 7–8 with a pH-stat, consumed 1 equiv. of alkali and gave an insoluble crystalline product having m.p. 185–189° (Found: C, 45.8; H, 5.6; N, 18.7. $C_{11}H_{14}N_4O_3S \cdot \frac{1}{2}H_2O$ requires C, 45.3; H, 5.2; N, 19.2%). The infrared spectrum had bands at 1747, 1695, 1667 and 1613 cm⁻¹. The same compound was obtained by treating the ester hydrochloride in methanol with an excess of triethylamine. Its insolubility and instability towards nucleophiles precluded further purification. Attempted recrystallization from boiling water afforded β -guanidino-L- α -toluene-*p*-sulphonamidopropionic acid having the correct analysis and infrared spectrum. Possible structures for the insoluble compound are discussed below.

Kinetics

The kinetics of hydrolysis of the substrates were followed with a pH-stat and recorder (Baines *et al.* 1964) and the

results were computed as described by Elmore, Kingston & Shields (1963). The kinetic mechanism was discussed by Baines *et al.* (1964), but some of the symbols used there have now been altered. The mechanism is summarized as follows:

The apparent zero-order rate constant, k_0 (formerly $k_{s(\text{app.})}$), is given by:

$$1/k_0 = 1/k_{+2} + 1/k_{+3}$$

The apparent Michaelis constant, K_m (formerly $K_{m(\text{app.})}$), is given by:

$$K_m = \frac{k_{+3}(k_{-1} + k_{+2})}{k_{+1}(k_{+2} + k_{+3})}$$

Our results indicated that the determination of the velocity of hydrolysis of trypsin-catalysed reactions could occasionally be biased by errors arising in the determination of enzyme concentration by the method of Green & Neurath (1953). Extinction values tended to be too high especially when very dilute solutions of enzyme were used. We found that the method of Lowry, Rosebrough, Farr & Randall (1951) was more reliable for determining the concentration of trypsin solutions. Calibration curves relating trypsin concentration and the extinction at 550 m μ and 750 m μ were constructed (Fig. 1). The concentration of

Fig. 1. Extinction values for trypsin solutions obtained by using the method of Lowry *et al.* (1951): ●, at 550 m μ ; ○, at 750 m μ .

each trypsin solution used to obtain the calibration curve was determined by the micro-Kjeldahl method (Miller & Houghton, 1945). The parameters of the calibration curves were computed by the method of least squares.

RESULTS AND DISCUSSION

Kinetics of trypsin-catalysed reactions. The kinetics of the hydrolysis of methyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate by trypsin at pH 8.0 have been determined at various temperatures (Table 1). With the higher homologues (II; $n = 3$ or 4), kinetic parameters were determined at pH 8.4 (Baines *et al.* 1964), but the buffering capacity of esters of γ -guanidino-L- α -toluene-*p*-sulphonamidobutyric acid was rather high at this pH and experimental traces on the pH-stat recorder were unsatisfactory. The pK_a value of the sulphonamide group is markedly dependent on the length of the side chain in this series (Table 2). The values of k_0 are likely to be nearly maximum at pH 8.0, however, and direct comparison can be made with the kinetic results for the higher homologues. The kinetics of hydrolysis of the methyl ester by trypsin were also studied at pH 7.0 and 25° to provide a direct comparison with α -*N*-toluene-*p*-sulphonyl-L-ornithine methyl ester (Baines *et al.* 1964). Lactamization of the latter substrate precluded a study of the kinetics of its hydrolysis

by trypsin at higher pH values. Finally, the kinetics of hydrolysis of propyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate by trypsin at pH 8.0 and 25° were determined.

From the results assembled in Table 1, a number of conclusions can be drawn. First, the values of k_0 are not significantly different for the methyl and propyl esters and it is likely that deacylation of the acyl-enzyme is rate-determining. This was found to be the case for the higher homologues (II, $n = 3, 4$) (Baines *et al.* 1964). Secondly, the K_m value varies with temperature in a complex manner, but this is to be expected since it is a parameter comprising four rate constants. Thirdly, methyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate is a much better substrate than α -*N*-toluene-*p*-sulphonyl-L-ornithine methyl ester under comparable conditions. The values of k_0 and K_m for the former are respectively 5.5-fold larger and 340-fold smaller than for the latter substrate. Nevertheless, the distance between the positively charged group in the side chain and the ester group is approximately the same for both compounds. The protonated guanidino esters (II), however, differ from the protonated amino esters (I) by having the positive charge delocalized. This may simplify the binding of a substrate when the chain length is a little less than optimum. This view is strengthened by comparing, on the one hand, the kinetic constants for the hydrolysis of methyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate and α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester and, on the other hand, the kinetic constants for the hydrolysis of the methyl esters of α -*N*-toluene-*p*-sulphonyl-L-ornithine and -L-lysine. Differences for the pair of compounds (II; $n = 2, 3$) are less marked than they are for the compounds (I; $n = 3, 4$). A side chain that is much shorter than optimum will either not span the distance between the appropriate groups in the enzyme or, if Koshland's (1958) theory of induced fit applies, will not be able to induce the conformational changes required in the enzyme. It is not surprising, therefore, that methyl β -guanidino-L- α -toluene-*p*-sulphonamidopropionate (II; R = CH₃, $n = 1$), like methyl γ -amino-L- α -toluene-*p*-sulphonamidobutyrate (I; R = CH₃, $n = 2$), is not a substrate for trypsin. Potential substrates for trypsin having side chains longer than optimum have been less extensively studied. Kitagawa & Izumiya (1959) found that ethyl α -*N*-benzoyl- γ -guanidinobutyrate is a better substrate than α -*N*-benzoylhomoarginine ethyl ester. Similarly, we have found that, though the Michaelis constants are similar for methyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate and α -*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester, the former substrate has a rather higher maximum velocity of hydrolysis.

Table 1. k_0 and K_m values for the trypsin-catalysed hydrolysis of esters of γ -guanidino-L- α -toluene-*p*-sulphonamidobutyric acid

Substrate (II; $n = 2$)	Values are given $\pm$ S.D.			
	pH	Temp.	k_0 (sec. ⁻¹)	$10^4 K_m$ (M)
R = CH ₃	7.0	25°	18.51 $\pm$ 0.55	4.54 $\pm$ 0.23
R = CH ₃	8.0	15	20.78 $\pm$ 0.16	4.82 $\pm$ 0.24
R = CH ₃	8.0	20	28.65 $\pm$ 0.22	6.63 $\pm$ 0.22
R = CH ₃	8.0	25	38.07 $\pm$ 0.40	6.94 $\pm$ 0.15
R = CH ₃	8.0	30	54.49 $\pm$ 1.12	7.68 $\pm$ 0.33
R = CH ₃	8.0	37	91.09 $\pm$ 0.79	13.13 $\pm$ 0.31
R = CH ₃ ·CH ₂ ·CH ₂	8.0	25	35.48 $\pm$ 0.85	5.31 $\pm$ 0.28

Table 2. pK_a values for the sulphonamide group in methyl esters of ω -guanidino- α -toluene-*p*-sulphonamido acids

Compound	pK_a	
	Spectrophotometric	Potentiometric
(II; R = CH ₃ , $n = 4$)	10.12 $\pm$ 0.16	10.29
(II; R = CH ₃ , $n = 3$)	9.88 $\pm$ 0.31	10.08
(II; R = CH ₃ , $n = 2$)	9.51 $\pm$ 0.12	9.44

Spectrophotometric values are given $\pm$ S.D.

Table 3. Activation constants for the trypsin-catalysed hydrolysis of methyl esters of ω -guanidino-L- α -toluene-*p*-sulphonamido acids

Values are given $\pm$ s.d.

Substrate (II; R = CH ₃)	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°)
$n = 4$	9.81 $\pm$ 0.07	9.22 $\pm$ 0.07	-24.8 $\pm$ 0.2
$n = 3$	10.37 $\pm$ 0.45	9.77 $\pm$ 0.45	-17.2 $\pm$ 1.5
$n = 2$	11.91 $\pm$ 0.42	11.32 $\pm$ 0.42	-13.2 $\pm$ 1.4
$n = 2$	12.00 $\pm$ 0.40*	11.41 $\pm$ 0.40*	-12.5 $\pm$ 1.3*

* In the presence of cholate (2 mM).

Activation constants for the trypsin-catalysed hydrolysis of methyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate, which were computed from the data in Table 1, are given in Table 3. For the series (II; $n = 2, 3, 4$), $\Delta H^\ddagger$ decreases and $\Delta S^\ddagger$ becomes more negative with increasing chain length. As discussed by Baines *et al.* (1964), the tendency for $\Delta S^\ddagger$ to become more negative as the chain length is increased may reflect an increased distortion of the molecular conformation, an increased binding of solvent or a more complex distribution of electrical charge in the transition state. We are inclined to the view that the conformation of the enzyme is subjected to increasing distortion in the ground state of the acyl-enzyme as the side chain of the substrate is lengthened beyond the optimum; this distortion would have to be overcome in the transition state, which has to accommodate those amino acid residues that are responsible for the catalytic process.

Measurement of k_0 for methyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate over a range of pH indicated that a group with $pK_{(app.)}$ 7.05 $\pm$ 0.02 must be dissociated in the intermediate acyl-enzyme for deacylation to proceed. The corresponding values for the hydrolysis of α -N-toluene-*p*-sulphonyl-L-arginine methyl ester and α -N-toluene-*p*-sulphonyl-L-homoarginine methyl ester are 7.85 $\pm$ 0.05 and 6.93 $\pm$ 0.04 respectively. The $pK_{(app.)}$ increases as k_0 increases for this series of substrates, and it is possible that this is a further reflection of conformational differences between the various acylated trypsin derivatives.

Kinetics of thrombin-catalysed reactions. Values of k_0 and K_m for the hydrolysis of the methyl and propyl esters of γ -guanidino-L- α -toluene-*p*-sulphonamidobutyric acid by thrombin at 25° are given in Table 4. Comparison with results reported by Curragh & Elmore (1964) shows that the values of k_0 and K_m for methyl γ -guanidino-L- α -toluene-*p*-

Table 4. k_0 and K_m values at 25° for the thrombin-catalysed hydrolysis of esters of γ -guanidino-L- α -toluene-*p*-sulphonamido acid

Values are given $\pm$ s.d.

Substrate (II; $n = 2$)	pH	$10^7 k_0$ (moles min. ⁻¹ enzyme unit ⁻¹)	$10^4 K_m$ (M)
R = CH ₃	7.0	1.758 $\pm$ 0.037	2.13 $\pm$ 0.21
R = CH ₃	8.0	2.107 $\pm$ 0.008	2.36 $\pm$ 0.13
R = CH ₃ ·CH ₂ ·CH ₂	8.0	0.400 $\pm$ 0.006	35.09 $\pm$ 1.06

sulphonamidobutyrate are approximately one-third and 15 times respectively those of α -N-toluene-*p*-sulphonyl-L-arginine methyl ester. It may be recalled that thrombin was found not to catalyse the hydrolysis of the homoarginine analogue. Thrombin thus resembles trypsin in being able to catalyse effectively the hydrolysis of substrates that have side chains containing one carbon atom less than the best substrate in a series and in manifesting a lower activity towards substrates containing side chains that are longer than optimum.

Unlike trypsin, however, thrombin hydrolyses the methyl and propyl esters of γ -guanidino-L- α -toluene-*p*-sulphonamidobutyric acid at quite different rates. Similar behaviour was observed in the thrombin-catalysed hydrolysis of esters of α -N-toluene-*p*-sulphonyl-L-arginine (Curragh & Elmore, 1964) and clearly, if the postulated mechanism is correct, acylation is partly rate-determining. For this reason, no attempt was made to determine activation constants for the thrombin-catalysed reaction. The effect of pH on the value of k_0 for the methyl ester was studied, however, and the results indicated that a group of $pK_{(app.)}$ 6.53 $\pm$ 0.02 must be dissociated for hydrolysis to proceed. This is in close agreement with the values found for other substrates of thrombin (Curragh & Elmore, 1964). The thrombin-catalysed hydrolysis of propyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate, like that of α -N-toluene-*p*-sulphonyl-L-arginine methyl ester, is inhibited by increasing ionic strength (Fig. 2). Since acylation is apparently partly rate-determining, this provides further evidence that it is the acylation step that is sensitive to change in ionic strength.

Effect of cholate on hydrolyses catalysed by trypsin and thrombin. The kinetics of thrombin-catalysed reactions are modified in the presence of cholate ion; the clotting of fibrinogen and the hydrolysis of α -N-benzoyl-L-arginine methyl ester are inhibited, whereas the hydrolyses of the methyl esters of α -N-toluene-*p*-sulphonyl-L-lysine and -L-arginine are accelerated (Curragh & Elmore, 1964). The effect of cholate on the hydrolysis of esters of γ -guanidino-L- α -toluene-*p*-sulphonamidobutyric

Fig. 2. Effect of salt concentration on the value of k_0 at 25° and pH 8.0 for the hydrolysis of propyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate (0.02M) by bovine thrombin.

acid by trypsin and thrombin has now been examined (Tables 5 and 6). With trypsin, cholate increases k_0 and decreases K_m (cf. Tables 1 and 5). The change in k_0 is not sufficient, however, to alter the activation constants significantly (Table 3). Qualitatively similar behaviour was observed for thrombin-catalysed reactions, but the decrease in K_m was more pronounced (cf. Tables 4 and 6). In the earlier study of the effect of cholate on the kinetics of thrombin-catalysed reactions (Curragh & Elmore, 1964), it was suggested that the effect of cholate was to decrease k_{+3} and to increase k_{+2} by a relatively large amount. This offered an explanation for the observation that cholate inhibited the hydrolysis of α -*N*-benzoyl-L-arginine ethyl ester where deacylation is rate-determining, but accelerated the hydrolysis of the methyl esters of α -*N*-toluene-*p*-sulphonyl-L-lysine and -L-arginine where k_{+2} and k_{+3} both contribute to k_0 . Moreover, by comparing the effect of cholate on the kinetics of hydrolysis of two esters of the same acid, evidence was obtained that k_{+2} was indeed affected by cholate. This hypothesis also explained the decrease in K_m in the presence of cholate. The effect of cholate on the thrombin-catalysed hydrolysis of esters of γ -guanidino-L- α -toluene-*p*-sulphonamidobutyric acid is explicable on the same basis. In the trypsin-catalysed hydrolysis of these esters, however, cholate clearly increases k_{+3} since deacylation is rate-determining for this system. The value of k_{+2} may also be increased by cholate and this could account for the small decrease in K_m . As pointed out in the earlier paper,

Table 5. k_0 and K_m values for the trypsin-catalysed hydrolysis of methyl γ -guanidino-L- α -toluene-*p*-sulphonamidobutyrate in the presence of cholate (2mM)

Values are given $\pm$ s.d.

pH	Temp.	k_0 (sec. ⁻¹)	$10^4 K_m$ (M)
7.0	25°	25.58 $\pm$ 0.25	4.34 $\pm$ 0.14
8.0	15	23.72 $\pm$ 0.27	3.96 $\pm$ 0.17
8.0	20	36.37 $\pm$ 0.37	4.94 $\pm$ 0.17
8.0	25	47.96 $\pm$ 0.77	5.10 $\pm$ 0.17
8.0	30	66.36 $\pm$ 0.96	6.96 $\pm$ 0.23
8.0	37	108.84 $\pm$ 1.30	10.79 $\pm$ 0.41

Table 6. k_0 and K_m values at 25° for the thrombin-catalysed hydrolysis of esters of γ -guanidino-L- α -toluene-*p*-sulphonamidobutyric acid in the presence of cholate (2mM)

Values are given $\pm$ s.d.

Substrate (II; $n = 2$)	pH	$10^7 k_0$ (moles min. ⁻¹ enzyme unit ⁻¹)	$10^4 K_m$ (M)
R = CH ₃	7.0	3.824 $\pm$ 0.006	0.557 $\pm$ 0.043
R = CH ₃	8.0	4.531 $\pm$ 0.011	0.553 $\pm$ 0.077
R = CH ₃ ·CH ₂ ·CH ₂	7.0	0.431 $\pm$ 0.004	—
R = CH ₃ ·CH ₂ ·CH ₂	8.0	0.456 $\pm$ 0.006	—

however, alterations to k_{+1} or k_{-1} or both would also affect K_m . Clearly, the effect of cholate is more complex than was originally envisaged and the observed effects are likely to differ for each enzyme-substrate combination.

Lactamization of methyl β -guanidino-L- α -toluene-*p*-sulphonamidopropionate. When an aqueous solution of methyl β -guanidino-L- α -toluene-*p*-sulphonamidopropionate (II; R = CH₃, $n = 1$) was maintained at pH 7.0 with alkali by using a pH-stat, a crystalline precipitate was obtained. The rate of reaction could be determined from the rate of addition of alkali and the reaction was considerably accelerated as the pH was increased to 8.0. The product had a melting point similar to that of β -guanidino-L- α -toluene-*p*-sulphonamidopropionic acid, but could be distinguished from that compound by means of its elementary analysis and infrared spectrum. The same compound was obtained when the ester hydrochloride was treated with an excess of triethylamine in methanol. The behaviour of this ester under basic conditions strongly resembles the ready conversion of α -*N*-toluene-*p*-sulphonyl-ornithine methyl ester and methyl γ -amino-L- α -toluene-*p*-sulphonamidobutyrate into lactams (Barrass & Elmore, 1957; Curragh & Elmore, 1962). The product from methyl β -guanidino-L- α -toluene-*p*-sulphonamidopropionate, however, is less stable

in aqueous solution than the lactams previously obtained from ω -amino- α -toluene- p -sulphonamido esters; after about three recrystallizations from hot water, it was completely converted into β -guanidino- L - α -toluene- p -sulphonamidopropionic acid. Two possible structures for the lactam are 1-amidino-3-toluene- p -sulphonamidoazetidin-2-one (III) and 5,6-dihydro-5-toluene- p -sulphonamidoisocytosine (IV), and the mechanism of lactamization presumably requires the loss of a proton from the conjugate acid (II; R = CH₃, n = 1) followed by cyclization to (III) or (IV). A definitive decision between the possible structures for the lactam is complicated by its insolubility and ease of hydrolysis. Thus far, we are inclined to favour structure (III), since the infrared spectrum contains a strong band at 1747cm.⁻². This is probably due to a carbonyl stretching mode, but its frequency is considerably higher than that expected for a five- or six-membered lactam. The presence of ring strain, which is suggested by the ease of hydrolysis, could account for the unusually high frequency of the

carbonyl band. In fact, the spectra of a large number of azetidinones have been shown to contain a strong absorption band in the region 1730–1760cm.⁻¹, and this is attributed to the carbonyl stretching mode (Thompson, Brattain, Randall & Rasmussen, 1949).

We are grateful to the Medical Research Council, to Imperial Chemical Industries Ltd. and to The Chemical Society for grants for equipment and chemicals. This work was supported by a research grant (HE-07062) from the U.S. Public Health Service.

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