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THE HYDROLYSIS OF α-*N*-ACETYLGLYCYL-L-LYSINE METHYL ESTER BY UROKINASE

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SUMMARY

1. The preparation of a sensitive, synthetic substrate for urokinase α-*N*-acetylglycyl-L-lysine methyl ester has been described.

2. The apparent K_m and v_{max} values for urokinase, thrombin (EC 3.4.4.13) and plasmin (EC 3.4.4.14) catalysed hydrolysis of this substrate have been determined.

3. Soybean trypsin inhibitor and Trasylol at concentrations which strongly inhibit the plasmin and trypsin catalysed hydrolysis of α-*N*-acetylglycyl-L-lysine methyl ester have been found to be without effect on urokinase or thrombin.

4. The K_i values for ε-aminocaproic acid and aminomethyl-cyclohexanoic acid which act as competitive inhibitors of the urokinase catalysed hydrolysis of α-*N*-acetylglycyl-L-lysine methyl ester have been determined at pH 7.0 and 7.85. The K_i for aminomethyl-cyclohexanoic acid at pH 7.00 is 10 times as large as the K_i for ε-aminocaproic acid at this pH but at pH 7.85 the K_i values for the two inhibitors only differ by a factor of 2. The implications of these findings are discussed.

INTRODUCTION

Urokinase, a proteolytic enzyme found in urine, has been shown to catalyse the conversion of plasminogen, the precursor of the blood proteolytic enzyme, to the active enzyme plasmin (EC 3.4.4.14)[1–5]. This reaction can also take place *in vivo* and urokinase is currently undergoing intensive clinical trial to assess its effectiveness as a thrombolytic agent.

The activity of a urokinase preparation may be expressed in terms of the rate at which it catalyses the activation of plasminogen under standard conditions. However, assays based on this reaction are complicated by the lack of precision and re-

Abbreviations: TAME, α-*N*-tosyl-L-arginine methyl ester·HCl; BAEE, α-*N*-benzoyl-L-arginine ethyl ester·HCl; AGLME, α-*N*-acetylglycyl-L-lysine methyl ester·acetate; ALME, α-*N*-acetyl-L-lysine methyl ester·HCl; CTNE, α-*N*-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester; Cbz-, carbobenzoxy-; SBTI, soybean trypsin inhibitor.

producibility of methods for determining plasmin and the problem of preparing reproducible plasminogen preparations[6].

As well as its ability to activate plasminogen, urokinase has a demonstrable proteolytic activity[5] and is also able to catalyse the hydrolysis of amino acid esters[5]. The principal problem in the use of methods based on proteolysis, or the hydrolysis of TAME or BAEE, for measuring urokinase is that relatively large quantities of the enzyme are required compared with the quantities needed in the plasminogen activator assays. Recently, two sensitive synthetic substrates for urokinase have been described: α-*N*-acetyl-L-lysine methyl ester[7] and carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester[8]. The former substrate has proved satisfactory in comparing the urokinase activity of preparations of widely different specific activities. Unfortunately, this compound has not been obtained in crystalline form. Although CTNE can be used for comparing the activity of urokinase preparations of high purity (>20 000 CTA* units/mg)[9], it is sensitive to enzymes which do not activate plasminogen but which contaminate cruder urokinase preparations[8].

For these reasons the synthesis of a sensitive, "specific", synthetic substrate for urokinase which could be obtained in crystalline form was undertaken. In this paper the preparation of a substrate which satisfied these requirements, α-*N*-acetylglycyl-L-lysine methyl ester, is described. AGLME can be obtained as a crystalline chromatographically homogeneous solid. Comparative kinetic data for the action of urokinase, plasmin, trypsin (EC 3.4.4.4) and thrombin (EC 3.4.4.13) on this substrate are reported. A comparison has been made of the inhibition of the esterase activities of these enzymes by Trasylol and Soybean trypsin inhibitor. The effect of the competitive inhibitors of plasminogen activation, ε-aminocaproic acid and aminomethyl-cyclohexanoic acid, on the urokinase catalysed hydrolysis of AGLME has also been studied.

EXPERIMENTAL

Materials

Unless otherwise stated the reagents used for this work were of analytical grade and were obtained from British Drug Houses Limited.

Substrates

Tosyl-L-arginine methyl ester·HCl (Koch-Light Limited).

Acetylglycyl-L-lysine methyl ester·acetate (I) was prepared by the following method which has been adapted from the method described for synthesis of α-*N*-tritylglycyl-L-lysine methyl ester by BOISSONAS *et al.*[10].

(a) *N*,*N'*-Di-Cbz-L-lysine (II) was prepared by either the method of KATCHALSKI[11] or BOISSONAS *et al.*[10] and was obtained in crystalline form; m.p. 77–80°.

(b) εCbz-L-Lysine methyl ester·HCl (III) was prepared from (II) by the method of BOISSONAS[10] and was twice recrystallized from anhydrous methanol–ether (1:10, v/v); m.p. 114–117°.

(c) α-*N*-Acetylglycyl-ε-Cbz-L-lysine methyl ester (IV). 5.15 g (44 mmoles)

* A CTA unit refers to the standard urokinase unit adopted by the Committee on Thrombolytic Agents, National Heart Institute, National Institutes of Health, Bethesda, Md. (U.S.A.). This unit is based on the activity of a standard urokinase preparation (CTA standard) prepared by this Committee.

acetylglycine and 9.5 ml (40 mmoles) of tri-*n*-butylamine were dissolved in 80 ml of dry tetrahydro-furan and the solution was cooled to −10°. 3.84 ml (40 mmoles) ethyl chloroformate were added drop-wise to this solution over a period of 20 min. A supersaturated solution of (III) was prepared by dissolving 13.5 g in a mixture of 9.5 ml (40 mmoles) of tri-*n*-butylamine and 40 ml dry tetrahydro-furan at 40° and rapidly cooling to 0°. This was mixed with the solution containing acetylglycine at 0° and the mixture stirred for 2 h at this temperature and a further 16 h at 20°. The solvent was then removed in a rotary vacuum evaporator and the oil obtained was dissolved in 400 ml ethyl acetate. This solution was twice extracted at 0° with water; 1 M HCl; 1 M NH_4OH and water. The remaining ethyl acetate solution was dried over anhydrous Na_2SO_4 for 16 h and crystals of *α-N*-acetylglycyl-*εN*-Cbz-L-lysine methyl ester were obtained on concentrating the solution, m.p. 105–106°.

(d) *α-N*-Acetylglycyl-L-lysine methyl ester acetate (I). The *εN*-Cbz group was removed from (IV) by hydrogenation at atmospheric pressure. 5 g (IV) were dissolved in 50 ml methanol containing 1 ml glacial acetic acid and 0.5 g of a 10% palladium-on-charcoal catalyst.

When hydrogenation was complete the catalyst was removed by filtration and the solvent by evaporation. The oil obtained was redissolved in anhydrous methanol and the solvent again removed. This process was repeated a further three times. The resultant oil was stored under dry ether until crystals formed. The product was recrystallized three times from hot methanol–ether (1:10, v/v).

The following analytical data were obtained: Calculated for $C_{13}H_{27}N_3O_6$; C, 48.14; H, 8.16; N, 12.7; found C, 48.10; H, 8.33; N, 12.6. m.p. 130–132° $[\alpha]_D^{23}$ −26.5 (in H_2O).

The recrystallized product showed only one spot when chromatographed on a thin layer of silica gel G using phenol–water (75:25, w/w) as developing solvent. Under these conditions, lysine, lysine methyl ester, acetylglycine and acetylglycyl lysine had mobilities different from AGLME.

Enzymes

Trypsin (crystalline, salt-free): Worthington Biochemical Corporation, Freehold, N.J., (U.S.A.) Lot No. TRL 6227;

Human thrombin standard Batch SB 66/126: American National Red Cross, Washington, D.C., (U.S.A.).

Streptokinase: Kabikinase - Kabi Pharmaceuticals Limited, London (Great Britain).

Human urokinase: Standard of the Committee on Thrombolytic Agents, National Heart Institute, Bethesda, Md. (U.S.A.).

Other samples of human urokinase were obtained from Abbott Laboratories and Pfizer Limited.

Human plasminogen: Prepared by a modification[12] of the KLINE procedure[13].

Inhibitors

Soybean trypsin inhibitor: Worthington Biochemical Corporation. 3 × recrystallized (Lot No. SI 5493).

ε-Aminocaproic acid: Koch-Light Limited, Colnbrook, Bucks. (Great Britain).

Aminomethyl-cyclohexanoic acid. The active isomer (Amikapron) was obtained

as a 10% aqueous solution from Kabi Pharmaceuticals Limited, London, (Great Britain).

Enzyme kinetic studies

Ester hydrolysis was followed by the titrimetric procedure in a pH stat, comprising a Radiometer TTT1C Titrator, SRBR2C Titrigraph, SBU1A syringe burette assembly, and a TTTA3 micro-titration assembly. Titrations were performed in a vessel maintained at 35±0.05° by means of a Haake thermostat. The titrand was $5 \cdot 10^{-2}$ M KOH and reactions were carried out in 0.15 M KCl. The volume titrated was 1.5 ml.

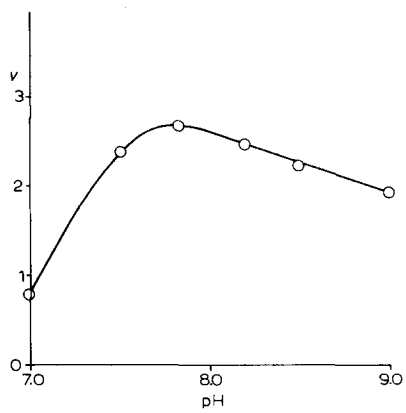
Enzyme solutions, unless otherwise stated, were prepared in gelatin diluent (0.5% gelatin–0.15 M KCl–$5 \cdot 10^{-4}$ M Tris (pH 7.0). Substrates and inhibitors were dissolved in 0.15 M KCl.

RESULTS

The hydrolysis of AGLME by urokinase

The effect of pH on the rate of urokinase-catalysed hydrolysis at $6 \cdot 10^{-3}$ M AGLME is shown in Fig. 1. The optimum was found at pH 7.80 and all further comparative data with the exception of some inhibition studies were obtained at this pH value.

At substrate concentrations greater than 10^{-3} M the rate of hydrolysis of ester followed zero-order kinetics until at least 10% of the substrate had been hydrolyzed. The reciprocal of the initial rates of hydrolysis plotted against the reciprocal of the substrate concentration produced a straight line (Fig. 2) which indicates the action



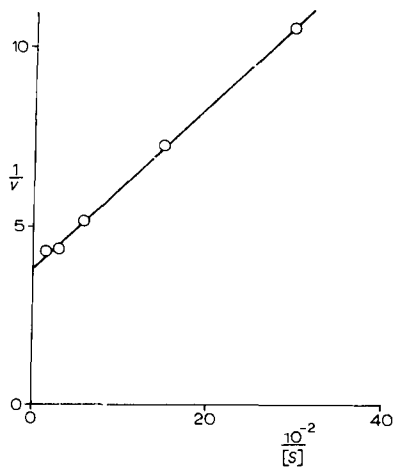


Fig. 1. The effect of pH on the rate of urokinase catalysed hydrolysis of AGLME. Ordinate, moles × 10^{-9} ester hydrolysed per min at 35° in solutions (1.5 ml) containing 100 CTA units urokinase per ml, $6 \cdot 10^{-3}$ M AGLME and 0.15 M KCl.

Fig. 2. Lineweaver and Burk plot of the effect of substrate concentration on the rate of AGLME hydrolysis by urokinase at pH 7.85. Ordinate, min mole^{-1} × 10^{-9}; abscissa, M^{-1} × 10^{-2}. Temp. 35° reaction in 0.15 M KCl (1.5 ml) containing 100 CTA units urokinase.

TABLE I

A COMPARISON OF THE APPARENT POTENCIES OBTAINED FOR UROKINASE PREPARATIONS OF DIFFERING SPECIFIC ACTIVITIES BY AGLME ESTERASE AND PLASMINOGEN ACTIVATOR ASSAYS

Results expressed as CTA units, obtained by comparison with CTA Standard. In the AGLME assay the activities are compared by measuring the rates of hydrolysis of AGLME at $6 \cdot 10^{-3}$ M, pH 7.85, in 0.15 M KCl and at 35°. In the plasminogen activator assay the comparisons are made using the diffusion assay of WALTON[15].

Urokinase sample	*Units/mg*		*Esterase*
	AGLME esterase assay	*Plasminogen activator assay*	*Activator*
A	355	379	0.94
B	715	715	1.00
C	3 780	3 540	1.06
D	13 000	12 500	1.04

of the enzyme on this substrate follows simple Michaelis–Menten kinetics[14]. The apparent K_m (K_m(app)) obtained from these data was $5.9 \cdot 10^{-4}$ M and the v_{max} $2.51 \cdot 10^{-2}$ μmole min^{-1} CTA unit^{-1}.

The correspondence between esterase activity and plasminogen activator activity of urokinase preparations of differing specific activity is shown by the results given in Table I. Samples obtained at various stages of the isolation of urokinase from a single batch of human urine were assayed by a quantitative fibrin plate method[15] (plasminogen activator assay) and the esterase method. A direct correlation was found between the values obtained by the two methods for urokinase preparations of specific activity; a range of 355 CTA units/mg to 13 000 CTA units/mg.

The action of trypsin, thrombin and plasmin on AGLME

AGLME proved to be an effective substrate for trypsin, thrombin and plasmin; as might have been expected from the known substrate specificity of these enzymes. The K_m (app) and v_{max} values obtained for thrombin and plasmin are given in Table II

The action of thrombin and plasmin appeared to follow Michaelis–Menten kinetics as shown by the linear curves obtained when the reciprocal of the rate of hydrolysis is plotted against the reciprocal of the substrate concentration (Figs. 3b

TABLE II

K_m (app) AND V_{max} VALUES OBTAINED FOR THE HYDROLYSIS OF AGLME BY THROMBIN, PLASMIN AND UROKINASE

Rates of ester hydrolysis were determined as described in METHODS at 35 ± 0.05°, pH 7.85, in solutions containing 0.15 M KCl.

Enzyme	*K_m (app)*	*v_{max}*
Thrombin	$3.3 \cdot 10^{-3}$ M	$2.76 \cdot 10^{-2}$ μmoles/min per NIH unit
Plasmin	$4 \cdot 10^{-3}$ M	3.34 μmoles/min per caseinolytic unit*
Plasmin (TAME substrate)	$8.2 \cdot 10^{-3}$ M	0.445 μmole/min per caseinolytic unit*
Urokinase	$5.9 \cdot 10^{-4}$ M	$2.51 \cdot 10^{-3}$ μmoles/min per CTA unit

* Caseinolytic units are the units described by SGAURIS[16].

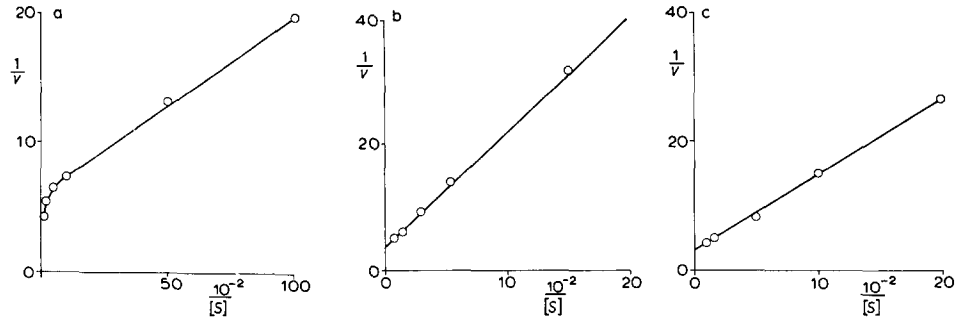


Fig. 3. Lineweaver and Burk plot of the effect of substrate concentration on the rate of hydrolysis of AGLME by trypsin (a), thrombin (b) and plasmin (c) at pH 7.85. Ordinate, min mole^{-1} × 10^{-9}. Abcissa, M^{-1} × 10^{-2}. Temp., 35°. Reaction in 0.15 M KCl (1.5 ml) containing (a) trypsin 10 μg/ml, (b) thrombin 6.67 NIH units/ml, (c) plasmin* 0.067 casein units/ml.

and c). Trypsin did not give rise to a linear plot and marked curvature at high substrate concentration (Fig. 3a) suggested activation of the enzyme by substrate. This is further illustrated by the shape of the curve obtained for the plot of $v/[S]$ against v (Fig. 4), which resembles the one given by TROWBRIDGE *et al.*[17], who demonstrated substrate activation of trypsin by high concentrations of TAME.

The nature of the bond split on the hydrolysis of ALGME by trypsin and urokinase

The possibility exists that the peptide bond as well as the ester bond in ALGME may be split during enzyme-catalysed hydrolysis. It was found however that after

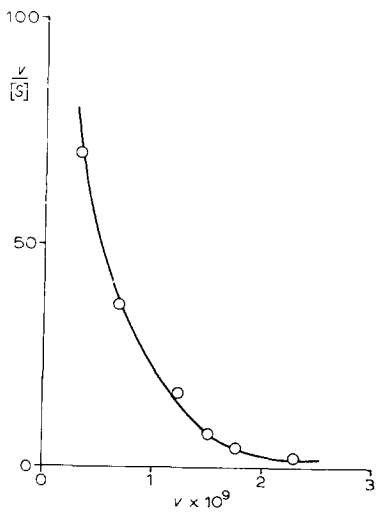


Fig. 4. Eadie plot of the effect of substrate concentration on the rate of AGLME hydrolysis by trypsin at pH 7.85. Ordinate, min^{-1} × 10^{-6}; abscissa, mole min^{-1} × 10^{9}. The data were derived from the experiment described in Fig. 3a.

* Plasmin prepared by mixing plasminogen (18 U./ml) with an equal volume of streptokinase (500 I.U./ml) in pH 7.0 buffer (Tris 0.005 M). After 10 min at 20° the mixture was diluted with a predetermined volume of 10^{-2} M HCl, to bring the pH to 3.0, and stored in ice until used.

prolonged hydrolysis of ALGME by trypsin or urokinase in the pH stat only one mole of acid was produced for each mole of substrate hydrolyzed. Furthermore, only one spot was found when the incubate was chromatographed on silica gel G coated plates using phenol–water (75:25, w/w) as eluting solvent. The spot was intermediate in mobility between AGLME and lysine methyl ester and did not correspond with lysine or acetylglycine, possible intermediates if the peptide bond had also been split. It is presumed that this spot is produced by acetylglycyl-lysine.

ε-Aminocaproic acid and aminomethyl-cyclohexanoic acid as competitive inhibitors of the urokinase catalysed hydrolysis of AGLME

LORAND AND CONDIT[9] found that ε-aminocaproic acid acted as a competitive inhibitor of the urokinase-catalysed hydrolysis of CNTE. The K_i (1–1.4 · 10^{-2} M) obtained at pH 7.0 was much higher than expected from the known effectiveness of ε-aminocaproic acid as a competitive inhibitor of plasminogen activation.

These experiments have been repeated on the urokinase-catalysed hydrolysis of ALGME using both ε-aminocaproic acid and aminomethyl-cyclohexanoic acid, a more effective inhibitor of plasminogen activation than ε-aminocaproic acid[18], at pH 7.0 and 7.85. The data obtained for the rate of hydrolysis of ALGME by urokinase at different substrate concentrations and containing either ε-aminocaproic acid at 3 · 10^{-2} M or aminomethyl-cyclohexanoic acid at 2.1 · 10^{-2} M at these two pH values are presented in graphical form in Figs. 5 and 6. The reciprocal plots for inhibited and uninhibited reactions extrapolate to the same maximal velocity indicating that the inhibition is competitive[14]. The K_i values obtained are given in Table III. The value for ε-aminocaproic acid at pH 7.0 (1.76 · 10^{-2} M) is slightly higher than that found by LORAND AND CONDIT[9] (1–1.4 · 10^{-2} M). The value of 1.58 · 10^{-3} M for aminomethyl-cyclohexanoic acid at this pH is in accord with the greater effectiveness of aminomethyl-cyclohexanoic acid as an inhibitor of plasminogen activation. The K_i values are both greater at pH 7.85 and the difference between aminomethyl-cyclohexanoic acid and ε-aminocaproic acid is a factor of two rather than an order of magnitude.

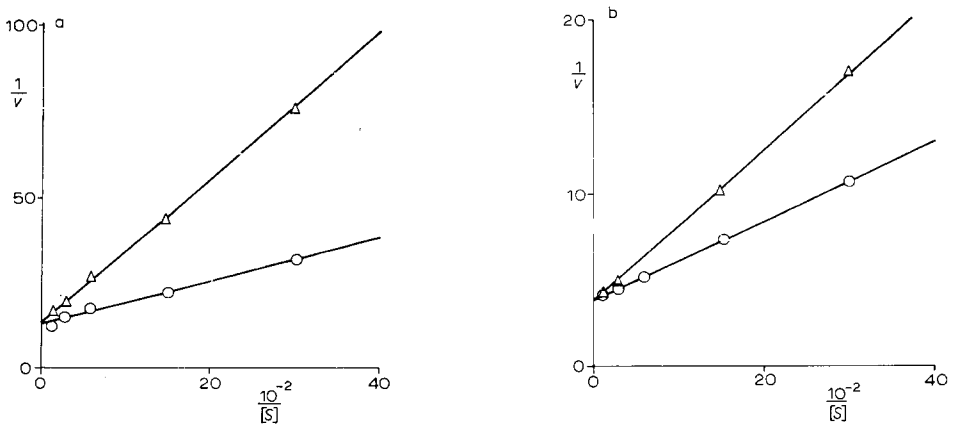


Fig. 5. Lineweaver and Burk plot of the effect of substrate concentration on the hydrolysis of AGLME by urokinase in the presence of 3 · 10^{-2} M ε-aminocaproic acid at pH 7.00 (a) and 7.85 (b). ○—○, without inhibition; △—△, in the presence of ε-aminocaproic acid. Abscissa, min mole^{-1} × 10^{-9}; ordinate, M^{-1} × 10^{-2}.

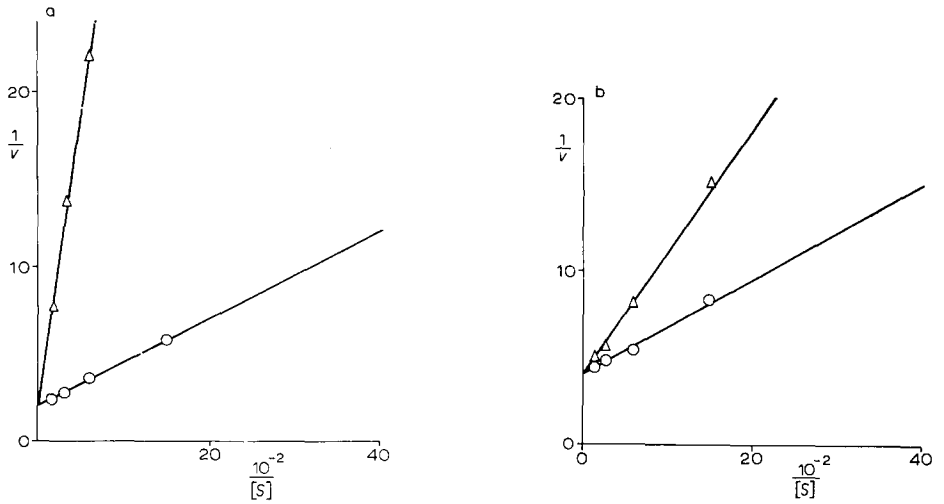


Fig. 6. Lineweaver and Burk plot of the effect of substrate concentration on the hydrolysis of AGLME by urokinase in the presence of $2.1 \cdot 10^{-2}$ M aminomethyl-cyclohexanoic acid at pH 7.00 (a) and 7.85 (b). ○—○, without inhibitor; △—△, in the presence of aminomethyl-cyclohexanoic acid. Abscissa, min mole^{-1} × 10^{-9}; ordinate, M^{-1} × 10^{-2}.

A comparison of the inhibitory effects of trasylol and soybean trypsin inhibitor on the hydrolysis of AGLME by thrombin, plasmin, trypsin and urokinase

LORAND AND CONDIT[9] compared the effect of the trypsin inhibitors SBTI and mingin[19] (urinary trypsin inhibitor) on the hydrolysis of CTNE by trypsin and urokinase. Urokinase was not affected by concentrations of these inhibitors which completely abolished trypsin activity. This work has been extended to study the effect of Trasylol (a polypeptide trypsin inhibitor isolated from bovine parotid glands which has been used for controlling hyperplasminaemic states)[20] and SBTI on the esterase activities of trypsin, urokinase, plasmin and thrombin.

The minimum concentrations of SBTI and Trasylol which would almost completely inhibit the esterase activity of trypsin were determined by prior experiment. Samples of thrombin, urokinase and plasmin diluted to contain approximately the

TABLE III

INHIBITOR BINDING CONSTANTS (K_i) OF ε-AMINOCAPROIC ACID AND AMINOMETHYL-CYCLOHEXANOIC ACID FOR THE UROKINASE CATALYSED HYDROLYSIS OF AGLME

K_i values were obtained using the relationship $K_i = \frac{i}{\frac{K_p}{K_m} - 1}$ where i is the inhibitor concentration and K_p the apparent Michaelis constant obtained for the urokinase catalysed hydrolysis in the presence of $2.1 \cdot 10^{-2}$ M aminomethyl-cyclohexanoic acid or $3 \cdot 10^{-2}$ M ε-aminocaproic acid. Experimental details are given with Figs. 5 and 6.

Inhibitor	K_i *(M)*	
	pH 7.0	*pH 7.85*
ε-Aminocaproic acid	$1.76 \cdot 10^{-2}$	$3.33 \cdot 10^{-2}$
Aminomethyl-cyclohexanoic acid	$1.58 \cdot 10^{-3}$	$1.21 \cdot 10^{-2}$

TABLE IV

THE EFFECT OF TRASYLOL AND SOYBEAN TRYPSIN INHIBITION ON THE AGLME ESTERASE ACTIVITY OF TRYPSIN, PLASMIN, THROMBIN AND UROKINASE

0.1-ml volumes of enzyme dissolved in 0.15 M KCl containing $5 \cdot 10^{-3}$ M Tris (pH 7.0) (trypsin, 10 μg/ml; plasmin*, 1 casein unit/ml; Thrombin, 150 NIH units/ml; urokinase, 700 units/ml) were mixed with an equal volume of inhibitor (5 μg/ml SBTI or Trasylol 50 units/ml**) in the same solvent. After 10 min at 20° the activity remaining was determined as described in METHODS. Substrate concentrations $6 \cdot 10^{-3}$ M AGLME.

Enzyme	*µmole ester hydrolysed per min*			*Inhibition (%)*	
	Enzyme + buffer	*Enzyme + Trasylol*	*Enzyme + SBTI*	*Trasylol*	*SBTI*
Trypsin	2.50	0.029	0.910	98.3	64
Plasmin	2.57	0.067	1.120	97.4	60
Thrombin	2.45	2.350	2.48	4.3	0
Urokinase	2.51	2.410	2.53	4.3	0

* Preparation described under Fig. 3.
** Manufacturer's units.

same ALGME esterase activity as the trypsin preparations were mixed with the predetermined quantity of SBTI or Trasylol. After incubation for 10 min at 20° the esterase activity remaining was determined and compared with the appropriate controls. The results are given in Table IV.

At these concentrations neither inhibitor affected urokinase activity or thrombin activity at concentrations which would inhibit the majority of the activity in trypsin or plasmin solutions.

DISCUSSION

The data presented by SHERRY *et al.*[7] on the urokinase-catalyzed hydrolysis of a series of α-*N*-acyl-substituted esters of arginine and lysine indicated that the nature of the acyl substituent profoundly affected the rate of hydrolysis. Acetyl derivatives proved to be much better substrates than benzoyl or tosyl compounds and it seems possible that steric factors play some part in the substrate specificity of this enzyme. Unfortunately, the most sensitive substrate, ALME, has not been crystallized from solvents and consequently cannot be obtained in the pure form necessary for studying the kinetics or for the routine standardization of this enzyme. Preparations made in this laboratory or obtained from elsewhere contained significant quantities of lysine, lysine-methyl ester and acetyl-lysine.

The introduction of a more polar but similarly small substituent group, acetylglycyl rather than acetyl, results in a compound which can be easily crystallized from methanol–ether (1:10, v/v) but possesses the desirable substrate properties of ALME. The thrice recrystallized material was found to be free from contaminating intermediates, such as acetylglycine, acetylglycyl-lysine, lysine or lysine methyl ester.

The K_m (app) value $5.9 \cdot 10^{-4}$ M found for ALGME as a substrate for urokinase is slightly lower than the value found by SHERRY[7] for ALME ($7.7 \cdot 10^{-4}$ M). The maxi-

mum velocity is also increased by a factor of 2.5. Using the pH stat with a modified titrand delivery system (syringe volume 0.15 instead of 0.5 ml) it is possible to measure as little as 50 CTA units of urokinase. The precision of the method is limited by the accuracy with which small samples of enzyme can be introduced into the reaction vessel and to some extent by the adsorption of the enzyme onto glass surfaces (M. MOZEN, personal communication).

The ester is not wholly specific for urokinase, but it is not hydrolyzed by the non-specific esterase contaminants in crude urokinase preparations, which have been shown to catalyse CTNE hydrolysis. It is a better substrate for plasmin than TAME which is commonly used to determine the esterase activity of this enzyme.

Discrimination between urokinase and trypsin or plasmin using this substrate may be made by taking advantage of the inhibitory effect of SBTI or Trasylol on the latter two enzymes. Thrombin, which is not susceptible to these inhibitors, can be distinguished from urokinase by the low rate of hydrolysis of ALGME. At $6 \cdot 10^{-3}$ M concentration the ratio of AGLME hydrolysis to TAME is 0.72:1 for thrombin whereas for urokinase the ratio is 78:1.

The high K_i values obtained by LORAND AND CONDIT[9] for the ε-aminocaproic acid inhibition of the esterase activity of urokinase have been confirmed. These values appear to increase with increasing pH. This effect is much more pronounced for aminomethyl-cyclohexanoic acid where the change in K_i from pH 7.0 to 7.85 is an order of magnitude. The pH values for the dissociation of the amino and carboxyl groups in ε-aminocaproic acid and aminomethyl-cyclohexanoic acid are 10.8, 4.37 (ref. 21) and 10.7, 4.35 (ref. 22), respectively. Thus the change in proportion of charged groups with pH would be almost identical for these two inhibitors.

Both molecules will be almost wholly in the form of "Zwitterions" (NH_3^+—R–COO^-) at pH values of 7–8 and the fractional change in number of charged or uncharged groups at the two pH values could hardly be expected to give rise to such a large change in K_i, as is the case for aminomethyl-cyclohexanoic acid. It is much more likely that the binding of inhibitor is affected by the dissociation of a group in the enzyme with a pK value of about 7.0 leading to some charge or conformational change of the inhibitor binding site.

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