Ester Hydrolysis by Urokinase*

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ABSTRACT: Urokinase preparations, varying in purity from 70,000 to 145,000 "Abbott fibrinolytic units" per mg of protein, show a constant relationship between fibrinolysis-promoting and ester-hydrolyzing activities. The latter was measured on the substrate *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester for which apparent Michaelis constants and turnover numbers were obtained at both *p*H 7 and 8. Urokinase may thus be properly regarded as a hydrolytic enzyme; its potency can be measured in a matter of a few minutes using relatively minute amounts of the enzyme. Ester hydrolysis by urokinase is not affected by either soybean or urinary trypsin inhibitor. Nor is it modified by L-1-tosylamido-2-phenylethyl chloromethyl ketone which is known to inactivate chymotrypsin. Both N^{α}-*p*-tosyl-L-arginine methyl ester and ε-aminocaproic acid, however, competitively inhibit the hydrolysis of the tyrosine ester by urokinase. A dissociation constant of about 10^{-2} was obtained for the complex of the enzyme with ε-aminocaproic acid at *p*H 7. The inhibition of urokinase-catalyzed hydrolysis by ε-aminocaproic acid provided an interesting study in that the latter not only served as an inhibitor but, by virtue of being a nucleophile, also contributed to the hydrolysis of the ester substrate.

Urokinase, a protein found in human urine, has the ability to convert the precursor of the fibrinolytic enzyme of blood plasma into the active enzyme plasmin. Therefore the potency of urokinase preparations is measured in terms of the activity of the plasmin produced. Since in its mode of action plasmin is quite similar to trypsin, these tests range from digestion of fibrin and casein to the hydrolysis of synthetic substrates (Ploug and Kjeldgaard, 1957; Remmert and Cohen, 1949; Troll *et al.*, 1954; Martin *et al.*, 1959). However, apart from being cumbersome, coupled assay systems clearly preclude a more penetrating investigation of the enzymatic properties of urokinase itself.

Recently we reported (Lorand and Mozen, 1964) that even highly purified urokinase preparations catalyzed the hydrolysis of *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (CTN),[1,2] but it could not be firmly concluded that the esteratic and the plasminogenic abilities (the latter measured by fibrinolytic assay) were necessarily related properties. Earlier reports (Kjeldgaard and Ploug, 1957) concerning the hydrolysis of esters by urokinase were based on considerably less pure enzyme preparations and thus permitted even less certain conclusions. The data given in the present paper show quite convincingly that, for urokinase preparations exceeding a certain level of purity, there exists a constant relationship between esteratic and "fibrinolytic" potencies. A unit of "fibrinolytic" activity is associated with a well-defined esteratic turnover number.

The direct hydrolysis of an ester by urokinase can be utilized to examine a number of problems which could not be unambiguously investigated in coupled enzyme assays. For example, ε-aminocaproic acid (EACA) was known to inhibit the combined urokinase-plasminogen-plasmin-fibrinolytic system (Alkjaersig *et al.*, 1959), but the immediate effect of EACA on urokinase itself could not be fully evaluated. We now find that EACA is a competitive inhibitor of urokinase.

After the present work had been completed, a publication by Sherry *et al.* (1964) gave an account of the hydrolytic activity of urokinase on yet another ester substrate, acetyl-L-lysine methyl ester. Their best enzyme preparation had a specific activity comparable to that described in the earlier paper of Lorand and Mozen (1964), and was about half as pure as the most active urokinase employed in the present work.

Materials and Methods

Urokinase preparations, of varying degrees of purity, were a gift from Abbott Laboratories, North Chicago, Ill. (W. White and M. M. Mozen, method of preparation to be published). Their plasminogenic potency was

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[1] The similarity of hydrolysis of CTN by a number of other enzymes (chymotrypsin, trypsin, thrombin; with seemingly different specificities for charged substrates) indicates that all these enzymes share a common specificity requirement when confronted with uncharged substrates (Lorand *et al.*, 1962). This property must be a direct consequence of the similarity of protein structure which forms the binding sites of these enzymes. The close structural identity between chymotrypsin and trypsin (Walsh and Neurath, 1964), especially in the peptide loops comprising the TPCK-sensitive histidine (Schoellman and Shaw, 1963; Hartley, 1964) and the active serine, illustrates the point.

[2] Abbreviations used in this work: CTN, *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; EACA, ε-aminocaproic acid; TAME, *p*-toluenesulfonyl-L-arginine methyl ester; STI, soybean trypsin inhibitor.

measured by a fibrinolytic assay, with one "Abbott fibrinolytic unit" of urokinase being equal to about 0.37 unit as defined by Ploug and Kjeldgaard (1957) and to 0.5 CTA unit (Committee for Thrombolytic Agents) as explained in Sherry *et al.* (1964).

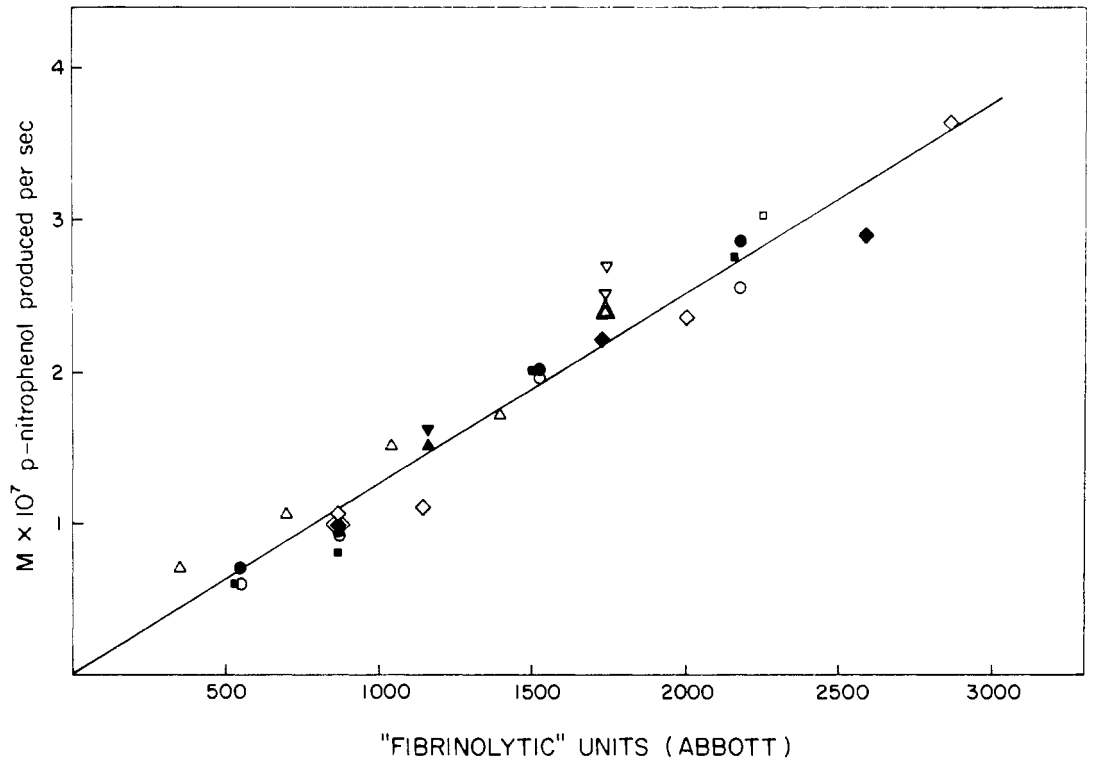

FIGURE 1: Relation between the *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl esterase and plasminogenic activities of urokinase preparations of varying purities. Ordinate denotes the initial velocity of ester hydrolysis at *p*H 8 (for details see text). Along the abscissa are the amounts of enzyme, in terms of fibrinolytic units, assayed for esterase activity. Over a period of 2 months three preparations of different purities were examined: 145,000 fibrinolytic units/mg protein (○, △, ▼, ●, ▲, ▽); 87,000 fibrinolytic units/mg protein (■, ◇, ◆); 70,000 fibrinolytic units/mg protein (□).

The *p-nitrophenyl* esters of *N-carbobenzoxy*-L-*tyrosinate* (CTN) and *acetate* were purchased from Mann Laboratories, New York City, and were dissolved in acetone prior to experimental use. *Trypsin* and *N^{α}-p-tosyl*-L-*arginine methyl ester* (TAME) were also products of Mann Laboratories. A trypsin (9700 benzoyl-arginine ethyl esterase units per mg) stock solution was made up into 0.001 M hydrochloric acid and was diluted 100-fold with 0.15 M sodium chloride just before use in the experiments. *Soybean trypsin inhibitor* (STI) was obtained from Armour and Co., Chicago. The *urinary trypsin inhibitor* Mingin was a gift from Dr. T. Astrup of Washington, D.C. Both of these inhibitors were dissolved into 0.15 M sodium chloride. *ε-Aminocaproic acid* (EACA) was purchased from Nutritional Biochemicals Corp., Cleveland, and the chymotrypsin-blocking reagent of Schoellman and Shaw (1963), L-*1-tosylamido-2-phenylethyl chloromethyl ketone* (TPCK), was obtained from Cyclo Chemical Corp., Los Angeles, Calif. *α-Chymotrypsin* (three-times crystallized) was a product of Worthington Biochemical Corp., Freehold, N.J.

Hydrolysis of CTN at *p*H 8 was carried out at 30°, using a Cary Model 11 automatic recording spectrophotometer for measuring the absorbancy changes associated with the production of nitrophenoxide ion at 400 mμ. The cuvet contained 2.4 ml of a 5:1 mixture of 0.15 M sodium chloride and 2-propanol, 0.4 ml of a 0.2 M Tris-HCl buffer of *p*H 8.0, 0.1 ml of urokinase dissolved in 0.15 M sodium chloride, and 0.1 ml of the ester substrate dissolved in acetone. The latter was added after the contents of the cuvet was allowed five minutes to come to temperature equilibrium. Initial enzyme-catalyzed rates were corrected for the non-enzymatic solvolysis of the ester which was measured by substituting 0.15 M sodium chloride for the urokinase solution in the test. Absorbancy changes were computed to nitrophenol concentration by using an $E_{1cm} = 1.6 \times 10^4$ liters/mole per cm.

Measurements at *p*H 7.0 were carried out in a similar manner. In this instance the concentration of nitrophenol was calculated by an E_{1cm} value of 10^4 liters/mole per cm.

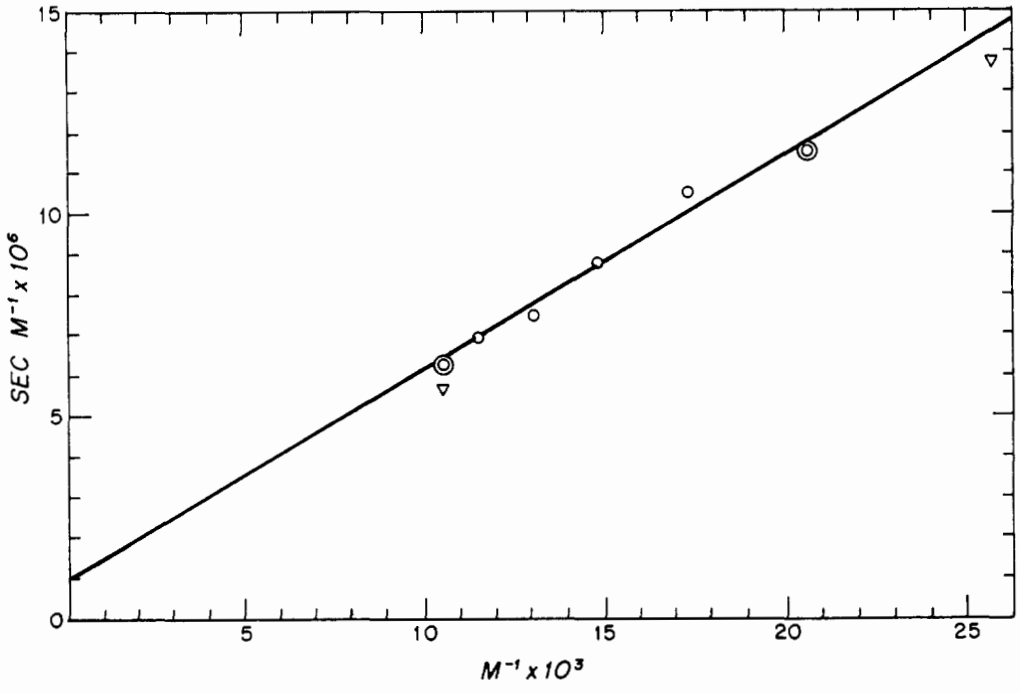

FIGURE 2: Lineweaver-Burk plot for the hydrolysis of *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester by urokinase at *p*H 8.0. The enzyme had a specific activity of 145,000 "Abbott fibrinolytic units" per mg of protein. Each experimental mixture contained 1160 units of urokinase. For details see text. The two sets of points (O, and ∇) were derived from different experiments. Ordinate, reciprocal initial velocity of ester hydrolysis; absicssa, reciprocal substrate concentration.

Results and Discussion

Relation between the Esteratic and Plasminogenic Potencies of Urokinase Preparations of Varying Purities. One of the logical methods for indicating the fact that two different biological activities reside in a single enzyme entity consists of demonstrating that a constant ratio between these activities holds at different states of purity of the enzyme. Figure 1 describes such a direct relationship between the esteratic and fibrinolysis-promoting activities of three urokinase preparations, the specific activities of which were 70,000, 87,000, and 145,000 "fibrinolytic units" per mg of protein, respectively. Various dilutions of the preparations were assayed for initial rates of CTN hydrolysis and the assays were carried out repeatedly over a period of 2 months during which the stock enzyme solution was stored in a deep freezer. It is seen that the enzyme retains its full activity during this storage. More important, however, is the finding that, regardless whether the 70,000- or 145,000-unit/mg protein preparation is tested, the same ratio between esteratic and fibrinolysis-promoting activities applies. Under the conditions of the experiment (slope of line in Figure 1), this ratio is 1.3×10^{-10} mole/liter per sec per "fibrinolytic unit." Our earlier observations (Lorand and Mozen, 1964) with considerably less pure urokinase preparations (500 and 14,000 units/mg of protein) indicated that these contained some esterase in addition to urokinase. Studies of ester hydrolysis with such urokinase preparations could lead only to fortuitous conclusions. However, when a degree of purity of about 70,000 units/mg of protein is achieved, esterase activity already seems to reflect the true enzymatic potency of the preparation.

Figure 2 shows a Lineweaver-Burk plot (Dixon and Webb, 1958) obtained at *p*H 8 for the esterase activity of the urokinase preparation of highest specific activity (145,000 units/mg of protein). The apparent Michaelis constant is 5×10^{-4} mole/liter and the reduced maximal initial velocity (turnover) is extrapolated to 8.6×10^{-10} mole/liter per sec per "fibrinolytic unit." Thus it is seen that the substrate concentration in the experiments given in Figure 1 permitted the reaction to proceed at a velocity about 6.5 times slower than maximal.

TAME as a Competitive Inhibitor of CTN Hydrolysis by Urokinase. The early reports concerning the hydrolysis of TAME by urokinase (Kjeldgaard and Ploug, 1957) were based on the use of rather impure preparations and, as pointed out here, could have easily been in error. It is known, however (W. White and M. M. Mozen, personal communication), that even highly purified urokinase, comparable to those reported in the present paper, had the ability of catalyzing the hydrolysis of TAME. In order to decide whether the hydrolysis of TAME and CTN by urokinase involved the same catalytic site, Lineweaver-Burk plots were measured for the initial velocity of hydrolysis of CTN (exactly as

in Figure 2) in the presence and absence of 10^{-4} M neutral TAME (dissolved into the 0.15 M sodium chloride), using the best available urokinase with a specific activity of 145,000 units/mg of protein. Since both lines were extrapolated to identical maximal initial velocities, it could be concluded that TAME competed with CTN for the enzyme. From the ratio of the slopes of the lines, an inhibition constant (K_I, given for the dissociation of [enzyme-TAME]; Dixon and Webb, 1958) of 2×10^{-4} mole/liter was calculated.

Ester Hydrolysis by Urokinase in the Presence of Known Inhibitors of Trypsin and Chymotrypsin. (1) The effect of soybean trypsin inhibitor (STI) on the hydrolysis of CTN by urokinase was examined at *p*H 8.0 under the conditions given in the methods section. In order to check the efficacy and time dependency of action of the inhibitor, the control experiments given in Table I were carried out first. As seen, STI interacted with trypsin to produce an 86% inhibition of enzyme activity after 5 minutes and a 92% inhibition after 10 minutes of incubation. In contrast, however, as shown in Table II, STI was totally ineffective vis à vis urokinase when the esteratic activity of urokinase was chosen to approximate that of trypsin. The additional observation that STI inhibited exactly the hydrolytic equivalent of trypsin, when added to a mixture containing both trypsin and urokinase, disposes of the possibility that some hypothetical contaminant in the urokinase preparation might have neutralized the effect of STI.

TABLE I: Inhibition of the Trypsin-catalyzed Hydrolysis of *N*-Carbobenzoxy-L-tyrosine *p*-Nitrophenyl Ester by Soybean Trypsin Inhibitor.[a]

Time of Incubation of Enzyme and Inhibitor Prior to Adding Substrate (min)	Initial Velocity of Ester Hydrolysis (M $sec^{-1} \times 10^8$)
(no inhibitor)	39
1	12
2	8.4
5	5.4
7.5	3.6
10	3.0

[a] Reactions were carried out at 30° and *p*H 8.0. Initial concentration of ester was 1.16×10^{-4} M. The 3-ml reaction mixtures contained 7.2×10^{-7} g of the enzyme and 6.6×10^{-7} g of the inhibitor, respectively.

(2) Mingin, a protein isolated from urine, is also known to be a powerful inhibitor of trypsin (Astrup, 1959). However, as seen in Table III, the hydrolytic activity of urokinase is not affected by Mingin.

TABLE II: Enzymatic Hydrolysis of *N*-Carbobenzoxy-L-tyrosine *p*-Nitrophenyl Ester in the Presence of Soybean Trypsin Inhibitor.[a]

Enzyme	Soybean Trypsin Inhibitor	Initial Velocity of Ester Hydrolysis (M $sec^{-1} \times 10^8$)
Urokinase		32
Trypsin		39
Urokinase + trypsin[b]		70
Urokinase	6.6×10^{-7} g	35
Trypsin	6.6×10^{-7} g	5.4
Urokinase + trypsin[c]	6.6×10^{-7} g	37

[a] Reactions were carried out at 30° and *p*H 8.0, with 5 minutes of incubation of enzyme and inhibitor prior to adding substrate. Initial concentration of ester was 1.16×10^{-4} M. The 3-ml reaction mixtures contained 1898 "fibrinolytic units" of urokinase (specific activity of 145,000 units/mg protein) and/or 7.2×10^{-7} g of trypsin as indicated in this table. [b] Trypsin was added immediately before admixing the nitrophenyl ester substrate. [c] Urokinase and soybean trypsin inhibitor were incubated first. Trypsin was added just prior to admixing of nitrophenyl ester.

(3) TPCK is a progressive irreversible inactivator of chymotrypsin, specifically alkylating an essential histidine moiety in the enzyme. It was deemed of interest to test whether TPCK would inactivate urokinase.

When a chymotrypsin solution was reacted with TPCK as described by Schoellman and Shaw (1963), the rate of CTN hydrolysis by the enzyme (1.5×10^{-11} mole) at *p*H 7 dropped from 42×10^{-8} to 4.5×10^{-8} mole/liter per sec. In contrast, 2000 "fibrinolytic units" of a urokinase preparation (specific activity of 22,000 units/mg protein) retained full activity toward CTN (15×10^{-8} mole/liter per sec) after treatment with TPCK in an identical manner.

Inhibition of Urokinase by ε-Aminocaproic Acid. As mentioned in the introduction, the direct hydrolysis of an ester substrate by urokinase lends itself particularly well to studying the immediate effects of possible inhibitors such as ε-aminocaproic acid (EACA).

(a) EACA AS A NUCLEOPHILIC CATALYST FOR THE HYDROLYSIS OF NITROPHENYL ESTERS. The appreciable nonenzymatic enhancement of hydrolysis of CTN observed in the presence of the large concentrations of EACA (*ca.* 10^{-2} M) which were to be examined brought up the possibility that EACA acted (by virtue of its amino function) as a nucleophile in general. In order to secure a quantitative appraisal along the lines indicated by

Jencks and Carriuolo (1960), the hydrolysis of *p*-nitrophenyl acetate was studied in the presence of EACA at *p*H 8.0. Employing the relationship:

$$\text{velocity} = k(\text{initial ester concn}) \cdot (\text{amine concn})$$

log k was calculated. The amine concentration was computed from the Henderson-Hasselbach equation by using a pK_a of 10.8 for the NH_3^+ in EACA (Edsall and Wyman, 1958). For example, in an actual experiment, with 0.13 M EACA and 1.4×10^{-4} M *p*-nitrophenyl acetate, a velocity of 4.14 moles/liter per min was observed for the hydrolysis of the ester under the conditions of Jencks and Carriuolo (1960). From this, a log $k = 3.16$ is derived which, when plotted against the pK_a of the NH_3^+ of EACA, fits very closely the line drawn by Jencks and Carriuolo (1960, their Figure 1) for nucleophilic catalysts. It is concluded therefore that the enhancement of ester hydrolysis by EACA can be fully attributed to its unprotonated amino function. It would then follow that at lower *p*H values, where the fraction of unprotonated amines is less, the rate of the EACA-catalyzed hydrolysis would be greatly reduced. Since this is obviously a desirable circumstance for examining the effect of EACA on urokinase, it was decided to study the hydrolysis of CTN by the combination of EACA and urokinase at *p*H 7.0.

TABLE III: Enzymatic Hydrolysis of *N*-Carbobenzoxy-L-tyrosine *p*-Nitrophenyl Ester in the Presence of the Urinary Trypsin Inhibitor Mingin.[a]

Enzyme	Mingin (g)	Initial Velocity of Ester Hydrolysis (M sec^{-1} $\times 10^8$)
Trypsin		39.5
Urokinase		29.5
Trypsin	2.8×10^{-6}	2.4
Trypsin	1.4×10^{-6}	8.1
Urokinase	2.8×10^{-6}	31.5

[a] Reactions were carried out at 30° and *p*H 8.0, with 5 minutes of incubation of enzymes and inhibitor prior to adding substrate. The initial concentration of ester was 10^{-4} M, and the 3-ml reaction mixtures contained either 1898 "fibrinolytic units" of urokinase (specific activity 145,000 units/mg protein) or 7.2×10^{-7} g of trypsin.

(b) EFFECT OF EACA ON THE UROKINASE-CATALYZED HYDROLYSIS OF CTN. (1) Theory. If hydrolysis was catalyzed by urokinase (U) in the absence of an inhibitor, a simple Michaelis-Menten relationship (Dixon and Webb, 1958) would describe the velocity (v_U) of the reaction:

$$v_U = \frac{k_{cat}(U)\cdot(CTN)}{(CTN) + K_M} \tag{1}$$

where k_{cat} is the catalytic constant, the symbols in brackets represent the concentrations of the corresponding reactants, and K_M is the apparent Michaelis constant of the urokinase reaction at *p*H 7.0.

The reaction occurring in the joint presence of urokinase and EACA may be analyzed into two components. On the one hand, EACA as a nucleophile would give a velocity contribution (v_{EACA}) of

$$v_{EACA} = k'\cdot(CTN)\cdot(EACA) \tag{2}$$

where k' is the nonenzymatic rate constant. On the other hand, EACA might be surmised to be a competitive inhibitor of urokinase so as to permit a reduced velocity (v_{iU}) for the inhibited enzymatic reaction (Dixon and Webb, 1958):

$$v_{iU} = \frac{k_{cat}(U)\cdot(CTN)}{(CTN) + K_M + \left(1 + \frac{(EACA)}{K_I}\right)} \tag{3}$$

where K_I would be the dissociation constant for the [urokinase-EACA] complex.

The sum of equations (2) and (3) would represent the total observed velocity (v_t) of the reaction in the presence of both urokinase and EACA. Therefore:

$$v_t - v_{EACA} = v_{iU} = \frac{k_{cat}(U)\cdot(CTN)}{(CTN) + K_M + \left(1 + \frac{(EACA)}{K_I}\right)} \tag{4}$$

Dividing equation (1) by equation (4):

$$\frac{v_U}{v_t - v_{EACA}} = \frac{k_{cat}(U)\cdot(CTN)}{(CTN) + K_M} \times \frac{(CTN) + K_M + \left(1 + \frac{(EACA)}{K_I}\right)}{k_{cat}(U)\cdot(CTN)} \tag{5}$$

This leads to:

$$\frac{v_U}{v_t - v_{EACA}} = 1 + \frac{(K_M/K_I)\cdot(EACA)}{(CTN) + K_M} \tag{6}$$

Inherent in the foregoing derivation was the assumption that EACA acted as a competitive inhibitor of urokinase. The validity of this could be tested, for example, by evaluating K_I over a range of EACA concentration while keeping that of CTN constant. The calculated values for K_I should be clearly independ-

TABLE IV: Hydrolysis of *N*-Carbobenzoxy-L-tyrosine *p*-Nitrophenyl Ester by Urokinase at Different Concentrations of ε-Aminocaproic Acid.[a]

EACA (M)	v_U	v_{EACA} (M sec^{-1} × 10^8)	v_t	Percentage Inhibition	K_I (M × 10^2)
0	14			0	
0.0013		0	13	7	0.9
0.0067		0	10	29	1.1
0.013		1	9	43	1.1
0.13		9	11	86	1.4

[a] Reactions were carried out at 30° and *p*H 7.0. For detailed explanation see text. Here v_{EACA} is the velocity of CTN hydrolysis by EACA, v_U is that for the uninhibited urokinase reaction, and v_t is the velocity measured in the presence of both urokinase and EACA. Percentage inhibition is defined as $100 - (v_t - v_{EACA})/v_U \times 100$. The K_I was calculated with the use of equation (6). As given in the text, $K_M = 2 \times 10^{-4}$ M. The concentration of CTN was 1.04×10^{-4} M.

ent of the concentration chosen for EACA. Experiments thus would have to be carried out to measure v_U, v_t, v_{EACA}, and K_M.

(2) Experimental mixtures comprised: 0.2 ml of varying proportions of 2 M EACA and 2 M sodium chloride (to keep the ionic strength constant); 1.9 ml of 0.15 M sodium chloride containing, when needed, 2088 "fibrinolytic units" of urokinase (specific activity of 145,000 units/mg protein); 0.4 ml of 0.1 M sodium phosphate buffer of *p*H 7.0; 0.4 ml 2-propanol; and 0.1 ml acetone containing the CTN substrate.

First, the K_M was obtained for the urokinase-catalyzed hydrolysis of CTN, in the absence of EACA. Using a Lineweaver-Burk plot similar to the one shown in Figure 2, a $K_M = 2 \times 10^{-4}$ mole/liter was computed from the measured slope and the extrapolated intercept of the line with the *y* axis (Dixon and Webb, 1958).[3]

Table IV summarizes the experiments which tested the validity of equation (6) as to whether EACA acted as a competitive inhibitor of urokinase. It will be readily noticed that even though the concentration of EACA was varied 100-fold, resulting in an inhibition of the enzymic reaction from 7 to 86%, the evaluated dissociation constants for the assumed [urokinase-EACA] complex remained practically unchanged (K_I = 0.9 to 1.4×10^{-2} mole/liter). This, of course, fully satisfies the assumption made at the outset that EACA is a competitive inhibitor of urokinase. The unusually high K_I, however, makes EACA appear a rather poor inhibitor and explains the necessity of having to use rather large concentrations of EACA for demonstrating its inhibitory effect. It is not known whether the EACA zwitterion ($^+H_3N \cdot (CH_2)_5 \cdot COO^-$) or the ($H_2N \cdot (CH_2)_5 \cdot COO^-$) anion form is the actual inhibitor of urokinase. In evaluating the K_I values from equation (6), the effective concentration was assumed to be the total concentration of EACA added.

[3] The reduced maximal velocity (turnover) at *p*H 7.0 came to 1.3×10^{-10} mole/liter per sec per "fibrinolytic unit." This is approximately seven times less than that measured at *p*H 8.0.

Acknowledgments

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