the course of ferricytochrome *c* reduction is generally very small.[4]

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[4] It will be noted that in the present investigation, the concentration of ferricytochrome *c*, 3×10^{-5} M, was always much smaller than that of acetaldehyde.

Kinetics of Transamidating Enzymes. Production of Thiol in the Reactions of Thiol Esters with Fibrinoligase[†]

C. G. Curtis, P. Stenberg, K. L. Brown, A. Baron, K. Chen, A. Gray, I. Simpson, and L. Lorand*

ABSTRACT: Fibrinoligase is a transamidating enzyme, formed from the "fibrin-stabilizing factor" or "factor XIII" zymogen in two distinct steps of activation, first by thrombin and then by calcium ions. The enzyme catalyzes both the hydrolysis and the aminolysis of specific thiol ester substrates, exemplified by β-phenylpropionylthiocholine. The steady-state velocity of formation of the thiol (*e.g.*, thiocholine) product, measured by a continuous direct reaction with 5,5′-dithiobis(2-nitrobenzoic acid), follows Michaelis-Menten kinetics yielding, for example, for the hydrolysis of β-phenylpropionylthiocholine at pH 7.5 and 25° an apparent Michaelis constant of about 0.3 mM and a turnover number of 0.2 $\sec^{-1}$. The kinetic analysis indicates that the pathway of catalysis includes an acyl-enzyme intermediate which can undergo deacylation by hydrolysis and also by aminolysis with an added amine substrate. The increased rate of thiol production in the presence of amines can be used as a quantitative index of specificity of the enzyme for various amine substrates. When kinetic experiments are carried out by mixing the thrombin-activated zymogen, calcium ions, and the ester substrate, without any prior incubation of the first two components, there is a marked delay in the onset of the steady-state formation of the thiol product. The lag phase corresponds to the calcium-dependent dissociation of the subunit structure of the thrombin-modified zymogen and it is linked to the unmasking of the active center of the transamidase. Calcium ions shorten the lag period and they also increase the steady-state rate of the enzymatic reaction. At low calcium concentration (5 mM), increase in general ionic strength accentuates these effects.

Following the demonstration that fibrinoligase (fibrin-stabilizing factor or blood coagulation factor XIII, activated by thrombin and by calcium ions) could catalyze the hydrolysis and aminolysis of certain thiol esters (Lorand *et al.*, 1972a), it became possible for the first time to analyze the kinetics of this transamidating enzyme by reactions involving exclusively synthetic substrates. Hitherto, such studies could be performed only in conjunction with protein substrates (see, *e.g.*, Lorand *et al.*, 1968, 1972b; Chung and Folk, 1972). The enzyme displays a great deal of specificity both toward the acyl portion and the thiol-containing residue in the esters, with β-phenylpropionyl- and *trans*-cinnamoylthiocholine among the best substrates examined so far giving an apparent Michaelis constant of the order of 10^{-4} M for hydrolysis. These compounds are also relatively stable in the absence of the enzyme, with a nonenzymatic rate constant for hydrolysis of about 10^{-6} $\sec^{-1}$ at pH 7.5 and 25°.

Continuous recording of the disappearance of substrates and

[†] From the Biochemistry Division, Department of Chemistry, Northwestern University, Evanston, Illinois 60201. *Received March 4, 1974.* This work was aided by U. S. Public Health Service Research Career Award HL-K6-3512 as well as by grants HL-02212 and HL-16346 from the National Institutes of Health. A Fulbright Travelling Fellowship to C. G. Curtis is gratefully acknowledged.

TABLE I: Enzymatic (E_0 = 0.16 μM) Hydrolysis of β-Phenylpropionylthiocholine, Measured at Different Initial Concentrations (a_0) of This Substrate in the Presence of Various Concentrations of Nbs_2, at 50 mM Calcium Chloride, pH 7.5, 25°.[a]

	$\lambda(Nbs_2)_0$, M $\times 10^6$			
	5	10	15	20
(a_0), M $\times 10^3$	v; M sec^{-1} $\times 10^{10}$			
0.108	186	170	185	186
0.255	248	253	254	250
0.500	290	309	309	300

[a] Velocities for thiol production (v) were obtained from the linear portions of progression curves (see Figure 1).

formation of products was considered an important requisite for detailed studies. The present paper deals with the kinetics of the enzymatic formation of the thiol product both in the purely hydrolytic reaction: $RCOSR' + H_2O \rightarrow RCOOH + R'SH$ and in combination with specific amine substrates of the enzyme: $RCOSR' + H_2NR'' \rightarrow RCONHR'' + R'SH$. A direct monitoring procedure (Ellman *et al.*, 1961) with 5,5′-dithiobis(2-nitrobenzoic acid) was used. Even though fibrinoligase is an SH enzyme (Curtis *et al.*, 1973), inclusion of this reagent in the reaction mixtures was still possible because the enzyme was protected against inactivation by the presence of the thiol ester substrates.

Materials and Methods

Fibrin-stabilizing factor (factor XIII) was prepared from citrated out-dated blood bank human plasma with slight modifications (Curtis *et al.*, 1974) of the procedure of Lorand and Gotoh (1970). The concentration of potential enzymatic sites, E_0, was measured by titration with [^{14}C]iodoacetamide following activation of the zymogen by thrombin and calcium ions as previously described (Curtis *et al.*, 1973, 1974).

For kinetic experiments, the proteolytic activation of the zymogen was achieved by mixing 1 mg of this protein with 3.75 NIH units of thrombin in 1 ml of 50 mM Tris-acetate buffer (pH 7.5) for 20 min at 25° (Curtis *et al.*, 1974). In the absence of calcium ions, the thrombin-modified zymogen could be kept on ice for a few hours.

β-Phenylpropionylthiocholine iodide was prepared by mixing the anhydride of β-phenylpropionic acid (1.00 g; 3.5 mmol, obtained from K & K Laboratories) with 2-dimethylaminoethanethiol (0.36 g; 3.4 mmol) at room temperature for 1 hr. 2-Dimethylaminoethanethiol was used in the base form and was prepared from the hydrochloride (Aldrich Chemical Co.) by repeated ether extraction from its sodium hydroxide neutralized aqueous solution, followed by distillation (56–59° at 63 mm). Acetone (10 ml) and then methyl iodide (1.0 g; 7.0 mmol) were added. After 2 hr the precipitated product was collected and washed with acetone and with anhydrous ether; it was then recrystallized from 1-propanol and also from absolute ethanol: yield 1.1 g (85%), mp 203–204° (uncor). *Anal.* Calcd for $C_{14}H_{22}NOSI$: C, 44.33; H, 5.85; N, 3.69. Found: C, 44.41; H, 5.90; N, 3.72.

trans-Cinnamoylthiocholine iodide was prepared as described for β-phenylpropionylthiocholine iodide from *trans*-cinnamic anhydride (1.39 g; 5.0 mmol) and 2-dimethylaminoethanethiol (0.53 g; 5.0 mmol) using dried benzene (15 ml) as solvent: yield 1.5 g (80%); mp 255° dec (recrystallized from water). *Anal.* Calcd for $C_{14}H_{20}NOSI$; C, 44.50; H, 5.35; N, 3.72. Found: C, 44.45; H, 5.56; N, 3.61.

2-Dimethylaminoethanethiol β-phenylpropionate hydrochloride was prepared by adding triethylamine (0.51 g; 5.0 mmol) and isobutyl chloroformate (0.68 g; 5.0 mmol) to a solution of β-phenylpropionic acid (0.80 g; 5.3 mmol) in anhydrous *p*-dioxane (70 ml). After 1 hr at room temperature, the mixture was filtered and 2-dimethylaminoethanethiol (0.45 g; 4.3 mmol in the base form) was added to the filtrate. After another hour at room temperature, hydrogen chloride in anhydrous diethylether was added to the solution until no further precipitation occurred. The product was then collected by filtration, washed with anhydrous diethyl ether, and recrystallized from absolute ethanol–anhydrous diethyl ether: yield 0.6 g (51%); mp 121–123° (uncor). *Anal.* Calcd for $C_{13}H_{19}NOS{\cdot}HCl$: C, 57.02; H, 7.36; N, 5.12. Found: C, 56.63; H, 7.31; N, 4.95.

2-Benzyldiethylaminoethanethiol cinnamate bromide (Ljunggren *et al.*, 1973) was a gift from Dr. Christine Ljunggren of the University of Uppsala, Sweden.

Amine substrates included *N*-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide or dansylcadaverine (Lorand *et al.*, 1968; Nilsson *et al.*, 1971), *N*-(5-aminopentyl)-*p*-toluenesulfonamide or tosylcadaverine (Lorand *et al.*, 1968; Stenberg *et al.*, 1972), *N*-(5-amino-3-thiapentyl)-5-dimethylamino-1-naphthalenesulfonamide or dansylthiacadaverine (Ljunggren *et al.*, 1974), *n*-butylamine hydrochloride (Eastman Kodak Co.), *N*-α-*p*-tosyl-L-lysine methyl ester hydrochloride (Cyclo Chemical Corp.), and putrescine dihydrochloride (Sigma).

In the hydrolysis or aminolysis of thiol esters, formation of the thiol product was assayed with the Ellman (1959) reagent, 5,5′-dithiobis(2-nitrobenzoic acid) or Nbs_2. Production of the yellow anion of 5-mercapto-2-nitrobenzoic acid ($\epsilon_{412} = 1.36 \times 10^4$ M^{-1} cm^{-1}) was monitored continuously using a Cary Model 16 recording spectrophotometer. All measurements were made at 25° in mixtures of 1.0 ml containing 50 mM Tris-acetate buffer (pH 7.5).

The calcium-dependent enzymatic reactions were measured by the inclusion of 5 mM EDTA and calcium chloride (as specified for each experiment) in the reference and test cuvets, respectively. In the hydrolysis experiments equal amounts of Nbs_2, then thio ester, were placed in each cuvet, followed by balancing for transmitted light. Finally, the thrombin-activated zymogen was added in identical amounts to the two cuvets. The protocol for aminolysis was similar, except for the inclusion of the amine substrates added to both cuvets just after the thiol ester.

For measuring nonenzymatic hydrolytic rates, both cuvets contained Nbs_2 but no thiol ester was added to the reference cell. Volumes were equalized with 50 mM Tris-acetate buffer (pH 7.5).

Results and Discussion

In general, the direct and continuous recording of thiol product formation in enzymatic reactions with Nbs_2 is possible only if the enzyme itself (*e.g.* cholinesterase; Ellman *et al.*, 1961) is insensitive to this reagent. With fibrinoligase, an SH enzyme (Curtis *et al.*, 1973), inclusion of Nbs_2 in the reaction mixtures was compatible with kinetic analysis because the enzyme was

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[1] Abbreviations used are: *N*-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide, dansylcadaverine; *N*-(5-aminopentyl)-*p*-toluenesulfonamide, tosylcadaverine; *N*-(5-amino-3-thiapentyl)-5-dimethylamino-1-naphthalenesulfonamide, dansylthiacadaverine; 1,4-diaminobutane, putrescine; toluenesulfonyl, tosyl; 5,5′-dithiobis(2-nitrobenzoic acid), Nbs_2.

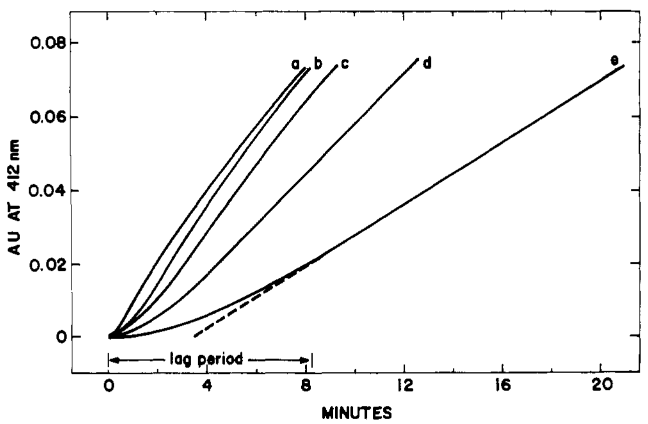


FIGURE 1: Progression curves for the formation of thiocholine in the fibrinoligase-catalyzed hydrolysis of β-phenylpropionylthiocholine iodide at 25° and pH 7.5. Reaction mixtures contained β-phenylpropionylthiocholine iodide (0.47 mM) and fibrinoligase (0.16 μM) at calcium chloride concentrations of (a) 100 mM, (b) 50 mM, (c) 30 mM, (d) 20 mM, and (e) 10 mM. Reduction of Nbs_2 shown on the ordinate in absorbancy units (AU) at 412 nm.

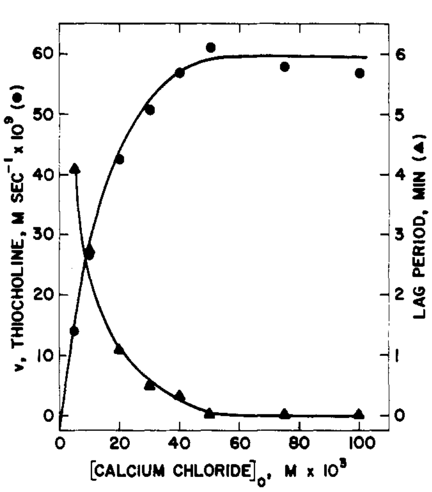


FIGURE 3: Effect of calcium ions on the formation of thiocholine from β-phenylpropionylthiocholine iodide (0.8 mM) in the presence of dansylcadaverine (1.0 mM) at pH 7.5, 25°. Reaction mixtures contained 0.09 μM of thrombin-activated zymogen.

protected against inactivation by the presence of the thiol ester substrate. As seen in Table I for the hydrolysis of β-phenylpropionylthiocholine, the measured steady-state velocities of the thiol product (even at the lowest concentration of thiol ester used) were independent of the initial concentration of Nbs_2 present in the cuvet. On the basis of these data, 10 μM of Nbs_2 were chosen for the monitoring of the reactions reported in this work.

Activation of fibrin-stabilizing factor (factor XIII) by thrombin is by itself not sufficient to unmask the cysteine side chain on the zymogen necessary for enzyme function (Curtis *et al.*, 1973, 1974). This occurs in an additional calcium ion dependent step and it is coupled to a dissociation of the heterologous subunit structure of the thrombin-modified zymogen (Lorand *et al.*, 1974). Using the experimental protocols given in the Methods section, whereby the thrombin-activated zymogen is added last to the calcium-containing reaction mixtures, the measurements would also reflect on the time-dependent generation of enzyme activity prior to a steady-state rate in substrate turnover. Progression curves for the enzymatic hydrolysis of β-phenylpropionylthiocholine at varying concentrations of calcium ions are shown in Figure 1. Indeed, it can be seen that the time required for developing full enzymatic activity, called the lag period, varied according to the concentration of calcium ions up to about 50 mM. This lag phase seems to correspond to the event of dissociation of the protomeric units

$$a'b \overset{Ca^{2+}}{\rightleftharpoons} a^* + b$$

which occurs coincident to the formation of the active center in a* (see Curtis *et al.*, 1973; Lorand *et al.*, 1974). Furthermore, since calcium ions control the above equilibrium and determine the fractional degree of generation of the a* enzyme (Curtis *et al.*, 1974), the linear rates following the lag phases also depend on calcium ions. At higher concentrations (>40 mM) of calcium ions, linearity is of rather short duration; this apparent deactivation of the enzyme may be attributed to a variety of circumstances, including the reported aggregation of a*.

An illustration of the dual role of calcium ions for shortening the lag phase and also increasing the steady-state rate of production of thiol is given in Figure 2 for the hydrolysis of β-phenylpropionylthiocholine. Similar relationships are noticed for the esterolytic reaction in the presence of an added amine substrate, dansylcadaverine, although the values obtained for the velocity of thiol liberation are higher (Figure 3).

The specific calcium ion effect may be accentuated by an increase of the general ionic strength (Figure 4). At a constant ionic strength of 0.2, as little as 4 mM calcium chloride sufficed

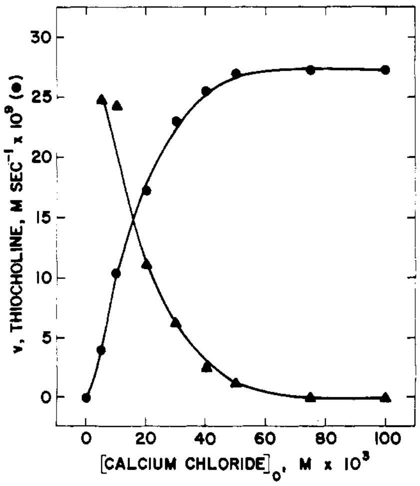


FIGURE 2: The dual effect of calcium ions on the lag period and on the steady-state hydrolysis of β-phenylpropionylthiocholine iodide (0.47 mM). Reaction mixtures contained 0.16 μM of thrombin-activated zymogen.

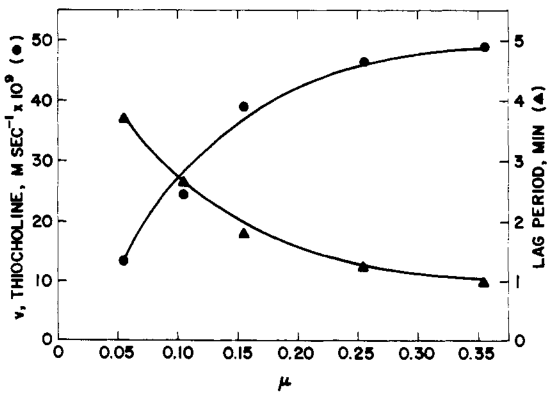


FIGURE 4: Influence of the general ionic strength (μ), adjusted by appropriate additions of sodium chloride to the 50 mM Tris-acetate buffer (pH 7.5), on the production of thiocholine for the reaction of 0.8 mM β-phenylpropionylthiocholine iodide with 1.0 mM dansylcadaverine at 5 mM calcium chloride. Mixtures contained 0.09 μM of thrombin-activated zymogen.

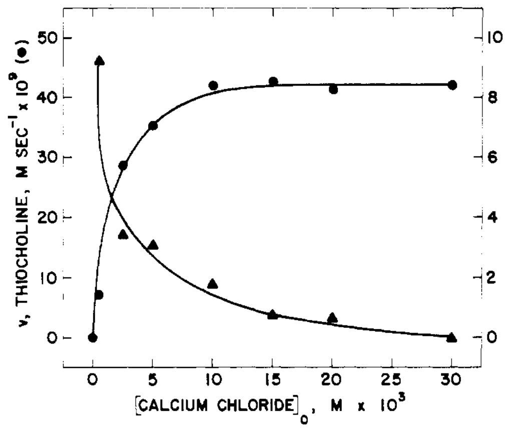


FIGURE 5: The specific effect of calcium ions at a constant ionic strength of 0.2 on the formation of thiocholine in the reaction of β-phenylpropionylthiocholine iodide (0.8 mM) with dansylcadaverine (0.8 mM) in the presence of 0.09 μM thrombin-activated zymogen.

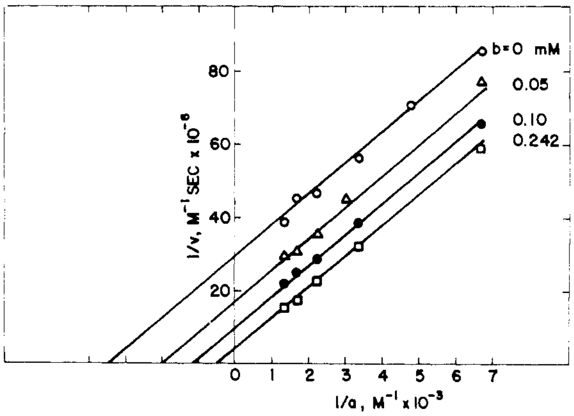


FIGURE 6: Double-reciprocal plots for the steady-state velocity of thiocholine formation at varying concentrations of β-phenylpropionylthiocholine iodide (a) and dansylcadaverine (b), at pH 7.5 and 25°, in the presence of 50 mM calcium chloride, at a fibrinoligase concentration of E_0 = 0.16 μM.

for generating half-maximal rate of enzymatic activity (Figure 5).

In considering the steady-state kinetics of the enzymatic reaction it is convenient first to discuss the results obtained in the purely hydrolytic system. The hydrolytic formation of thiols from all ester substrates presented in this work was shown to obey Michaelis-Menten kinetics. The Lineweaver-Burk plot for the hydrolysis (abbreviated as hyd in symbols) of β-phenylpropionylthiocholine is given as illustration in Figure 6 (top line without any amine added; *i.e.*, $b = 0$), from which an apparent Michaelis constant $K^{hyd}{}_{m,app} = 3 \times 10^{-4}$ M and a $k^{hyd}{}_{cat}$ of 0.2 sec^{-1} were calculated. Using the assumption that the model for the pathway of ester hydrolysis comprises two consecutive intermediates of the enzyme (E-SH)

$$R_2COSR_1 + HS{-}E \underset{}{\overset{K_a}{\rightleftharpoons}} [R_2COSR_1; HS{-}E] \xrightarrow[\text{acylation}]{k_2} R_2COS{-}E + HS{-}R_1(P_1) \quad (1)$$

$$R_2COS{-}E + H_2O \xrightarrow[\text{hydrolysis}]{k_3} HS{-}E + R_2COOH(P_2) \quad (2)$$

with P_1 and P_2 representing the two products and K_a as the dissociation constant of the enzyme-substrate complex, the rate equations yield $K^{hyd}{}_{m,app} = K_a k_3/(k_2 + k_3)$ and $k^{hyd}{}_{cat} = k_2/k_3/(k_2 + k_3)$ (*e.g.*, Kézdy and Kaiser, 1970). A comparison of β-phenylpropionylthiocholine with 2-dimethylaminoethanethiol β-phenylpropionate (Table II) revealed no significant difference either in $K^{hyd}{}_{m,app}$ or in $k^{hyd}{}_{cat}$ even though their nonenzymatic rates differed by a factor of about 45. Values of $k^{hyd}{}_{cat}$ for the *trans*-cinnamoylthiocholine and 2-benzyldiethylaminoethanethiol *trans*-cinnamate were found to be identical even though the latter underwent nonenzymatic hydrolysis about six times faster. Taken as pairs, these two sets of substrates represent compounds with different leaving groups; hence the results would be consistent with the idea that in the enzymatic reaction deacylation would be the rate limiting step (*i.e.*, $k^{hyd}{}_{cat} = k_3$). However, since the increased nonenzymatic hydrolytic rate of the 2-dimethylaminoethanethiol ester over the thiocholine ester is attributed to intramolecular catalysis (Hansen, 1963; Bruice and Benkovic, 1966) rather than to a better leaving group, one questions the validity of this example. The sixfold increase of nonenzymatic rate for the 2-benzyldiethylaminoethanethiol leaving group as compared to thiocholine in the *trans*-cinnamate substrates may actually not be large enough to permit an unambiguous conclusion with respect to the enzymatic reaction. Thus the issue as to whether the fibrinoligase-catalyzed hydrolysis of thiol esters is acylation or deacylation rate limiting must remain open. It could be mentioned that, in general, with a number of enzymes (chymotrypsin, trypsin, papain; see Bruice and Benkovic, 1966; Jencks, 1969; Bender, 1971) it appears that hydrolysis of the thermodynamically more stable esters and amides ($-\Delta G°{}_{hyd,pH\ 7} \approx$

TABLE II: Kinetic Constants for the Nonenzymatic and for the Fibrinoligase-Catalyzed Steady-State Hydrolysis of Thiol Esters at pH 7.5 and 25°.

		Enzymatic		
	Nonenzymatic sec^{-1} × 10^6	$K^{hyd}{}_{m,app}$ M × 10^3	$k^{hyd}{}_{cat}$ sec^{-1}	$k^{hyd}{}_{cat}/K^{hyd}{}_{m,app}$ M^{-1} sec^{-1} × 10^{-3}
$C_6H_5(CH_2)_2COS(CH_2)_2N(CH_3)_3{}^+I^-$ β-Phenylpropionylthiocholine iodide	1.3	0.29	0.21	0.74
$C_6H_5(CH_2)_2COS(CH_2)_2N(CH_3)_2 \cdot HCl$ 2-Dimethylaminoethanethiol β-phenylpropionate hydrochloride	58.5	0.18	0.13	0.74
$C_6H_5CH{=}CHCOS(CH_2)_2N(CH_3)_3{}^+I^-$ *trans*-Cinnamoylthiocholine iodide	1.3	0.10	0.08	0.80
$C_6H_5CH{=}CHCOS(CH_2)_2N(CH_2C_6H_5)(C_2H_5)_2{}^+Br^-$ 2-Benzyldiethylaminoethanethiol *trans*-cinnamate bromide	8.0	0.25	0.08	0.32

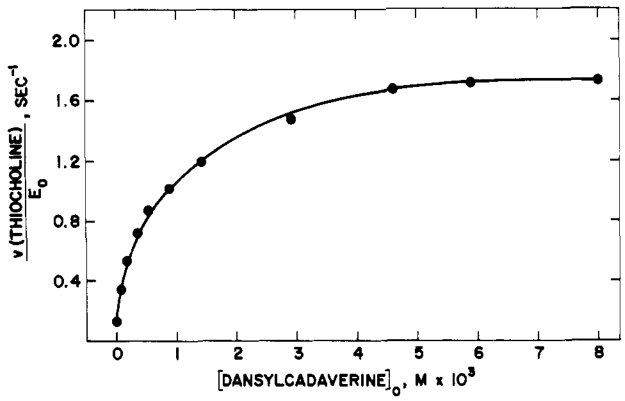
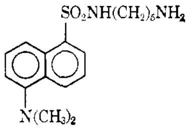
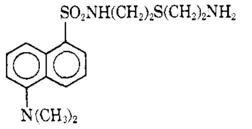
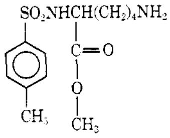


FIGURE 7: Enhancement of the steady-state formation of thiocholine by various concentrations of dansylcadaverine, added to 1.5 mM β-phenylpropionylthiocholine iodide at 50 mM calcium chloride and E_0 = 0.12 μM.

TABLE III: Enhancement of the Steady-State Formation of Thiocholine in the Reactions of Five Different Amines with 1.5 mM of β-Phenylpropionylthiocholine Iodide, at 50 mM Calcium Chloride, E_0 = 0.12 μM, pH 7.5, and 25°.[a]

Amine Substrates	$v^{am}{}_{lim}/E_0$ sec^{-1}	$K^{am}{}_{m,app}$ M × 10^4
 *N*-(5-Aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide; dansylcadaverine	1.7	8
 *N*-(5-Amino-3-thiapentyl)-5-dimethylamino-1-naphthalenesulfonamide; dansylthiacadaverine	1.7	4
 *N*-α-*p*-Tosyl-L-lysine methyl ester	1.4	11
$NH_2(CH_2)_4NH_2$ Putrescine	2.1	77
$CH_3(CH_2)_3NH_2$ *n*-Butylamine	1.2	430

[a] Limiting aminolytic enhancement rates ($v^{am}{}_{lim}$) were calculated from Lineweaver–Burk transforms of data (such as shown in Figure 7) after subtracting the purely hydrolytic velocity. Amine concentrations required to bring about half of the limiting velocities are denoted as $K^{am}{}_{m,app}$.

2-5 kcal mol^{-1}) proceeds with acylation being rate limiting, whereas active nitrophenyl esters and acylimidazoles ($-\Delta G°_{hyd,pH\ 7} \approx$ 10-12 kcal mol^{-1}) are hydrolyzed with deacylation as the slowest step. Thiol esters ($-\Delta G°_{hyd,pH\ 7} \approx$ 6-7 kcal mol^{-1}), such as the ones used in the present investigation, would occupy an intermediate position of stability (Jencks, 1968).

In the presence of specific primary amines, fibrinoligase is known to catalyze the formation of amides from the thiol ester substrates (Lorand *et al.*, 1972a). The enzyme kinetics of amide production will be the subject of a separate paper and the present work focuses only on the increased steady-state rate of formation of the thiol product with amines added. Velocity enhancements by various concentrations of dansylcadaverine, one of the most specific amine substrates of the enzyme (Lorand *et al.*, 1968), are shown in Figure 7, using 1.5 mM β-phenylpropionylthiocholine which represents approximately 6 × $K^{hyd}{}_{m,app}$. The saturation type of curve indicates an equilibrium of the amine with some form of the enzyme and suggests that, for the reaction with amines, eq 3 should be included as an alternate pathway to eq 2 for deacylation

$$R_2COS{-}E + H_2NR_3 \overset{K_b}{\rightleftharpoons} [R_2COS{-}E; H_2NR_3] \xrightarrow[\text{aminolysis}]{k_5} HS{-}E + R_2CONHR_3(P_3) \quad (3)$$

where K_b is the dissociation constant for the acyl-enzyme–amine complex.

From experiments such as that shown in Figure 7, the maximally obtainable rate of thiol production as well as the amine concentration required to bring about half of this maximal velocity could be estimated. Table III summarizes the results for the reaction of 1.5 mM β-phenylpropionylthiocholine with five different amines at pH 7.5 and 25°. Three of the amines, dansylcadaverine (Lorand *et al.*, 1968), dansylthiacadaverine (Ljunggren *et al.*, 1974), and *N*-α-*p*-tosyl-L-lysine methyl ester, are considered to be very good substrates for the enzyme; the fourth, putrescine (Lorand *et al.*, 1972b), is of intermediate and the fifth, *n*-butylamine (Lorand *et al.*, 1968), is of low specificity. While the concentration of amines necessary for half-maximal velocity of thiocholine production varied by more than two orders of magnitude within this series, the maximally obtained rates at saturating concentrations of all amines were essentially the same (1.2-2.1 sec^{-1}). This latter finding could be interpreted to indicate that, as amines are added, acylation (k_2) becomes the rate-limiting step.

The effects of varying the concentrations of both the thiol ester and the amine in the enzymatic reaction are illustrated in Figure 6 for the β-phenylpropionylthiocholine–dansylcadaverine substrate pair. Reciprocal ester concentrations are plotted against reciprocal velocities of thiol production for different concentrations of dansylcadaverine. Inasmuch as the slopes of the lines are independent of amine concentration and actually are the same as found for the purely hydrolytic reaction, the results are in agreement with the formulation of the reaction pathway given by eq 1-3. The steady-state solution of the appropriate rate equations for the formation of the thiol product yields

$$\frac{E_0}{v} = \frac{K_a}{k_2}\frac{1}{a} + \frac{(k_2 + k_3)K_b + (k_2 + k_5)b_0}{k_2k_3K_b + k_2k_5b_0}$$

and it is seen that if the concentration of amine b_0 = 0, the slope of the line K_a/k_2 still remains the same and the intercept becomes $(k_2 + k_3)/k_2k_3$, *i.e.* $1/k^{hyd}{}_{cat}$.

Similar sets of parallel Lineweaver–Burk lines were obtained also for the reactions of β-phenylpropionylthiocholine with tosylcadaverine and *n*-butylamine. The fact that none of the amines caused a change in the slopes of these lines could possibly be interpreted to mean that neither the binding of the ester substrate to the enzyme (measured by K_a) nor the rate of transesterification (k_2) in forming the acyl-enzyme intermediate is actually affected by the presence of an added amine.

The Lineweaver–Burk type of presentation (Figure 6) offered yet another means of evaluating the specificity of the enzyme toward amine substrates. At low concentrations of amines (b_0), reciprocals of the intercepts on the vertical axis (*i.e.*, the limiting velocities, $v^{am}{}_{lim}$, as $a \rightarrow \infty$) were found to vary in a linear manner: $v^{am}{}_{lim} = Cb_0 + v^{hyd}{}_{lim}$. The last term is the maximal velocity of ester hydrolysis in the absence of added amine and C is a characteristic constant for a particular amine substrate. The linear relationship was verified for dansylcadaverine (<0.25 mM), tosylcadaverine (<0.33 mM) and butylamine (<6 mM) which gave C values of 8×10^{-4}, 1.5×10^{-4}, and 5.2×10^{-6} sec^{-1} respectively. Clearly, a larger C represents a higher aminolytic enhancement factor and, since the three amines have nearly identical pK_a's, the relative order of the constants within this series can be assumed to be an expression of the specificity of the enzyme for the side chains of the amine substrates themselves. It should be recalled that virtually the same relative order of dansylcadaverine:tosylcadaverine:butylamine = 160:30:1 was established previously for the potencies of these compounds for inhibiting the cross-linking of fibrin in bioassays (Lorand *et al.*, 1968; Lorand and Nilsson, 1972).

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