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Transamidase Kinetics

AMIDE FORMATION IN THE ENZYMIC REACTIONS OF THIOL ESTERS WITH AMINES

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1. β-Phenylpropionylthiocholine and *N*-(5-aminopentyl)-5-dimethylaminonaphthalene-1-sulphonamide (dansylcadaverine) serve as a pair of water-soluble (pH7.5) model substrates for transamidating enzymes. Amide formation could be followed directly through fluorescence measurements by monitoring the continuous extraction of the water-insoluble coupling product, *N*-(β-phenylpropionyl)dansylcadaverine, into *n*-heptane. By this procedure, the steady-state kinetics of glutamine–lysine endo-γ-glutamyltransferase from human plasma (fibrinoligase, thrombin- and Ca^{2+}-activated blood coagulation Factor XIII) and from guinea-pig liver (liver transglutaminase) were investigated at 25°C. 2. With β-phenylpropionylthiocholine as the varied substrate, Lineweaver–Burk plots with various concentrations of dansylcadaverine intercept on the horizontal axis, suggesting that formation of the acyl-enzyme is rate limiting. 3. On the basis of functional normality of active sites, $k_{cat.}$ values of $1.8 s^{-1}$ and $0.9 s^{-1}$ were obtained for the plasma and liver γ-glutamyltransferase respectively. The two enzymes show identical affinities for the first substrate, β-phenylpropionylthiocholine, with K_a 4×10^{-4} M. 4. Utilization of the second substrate, dansylcadaverine, appears to be an order of magnitude more efficient with the liver enzyme. 5. *N*-(5-Amino-3-thiapentyl)-5-dimethylaminonaphthalene-1-sulphonamide (dansylthiacadaverine) could be used instead of dansylcadaverine in the fluorescent kinetic system. 6. Competitive inhibition by a non-fluorescent amine substrate histamine was also evaluated.

The enzymes used in this study may be regarded as Ca^{2+}-dependent acyltransferases which catalyse the formation of γ-glutaminyl-ε-lysyl bridges between a glutaminyl acceptor and a donor lysine and also the incorporation of amines into proteins (e.g. putrescine into casein). In plasma, the active enzyme (fibrinoligase, FSF* or factor $XIII_a$) is generated at the time of blood clotting from a zymogen precursor (fibrin-stabilizing factor, FSF or factor XIII) for the purpose of catalysing the formation of covalent bridges between fibrin units to increase the elasticity of the clot network (see Lorand, 1972). However, the functions of the other two related enzymes used in this investigation, namely, thrombin-activated platelet factor XIII (see McDonagh & Wagner, 1972) and guinea-pig liver transglutaminase (see Folk, 1972) are not known. Nevertheless, because of the confusing nomenclature these enzymes could be referred to by the systematic name glutamine–lysine endo-γ-glutamyltransferases and the individual enzymes by the trivial names, plasma (or platelet or liver) endo-γ-glutamyltransferase.

The introduction of water-soluble thiol esters provided an impetus for studying the action of plasma γ-glutamyltransferase in fully synthetic substrate systems (Lorand *et al.*, 1972*c*). In many respects, thiol esters could be used more conveniently for steady-state kinetic studies than *p*-nitrophenyl esters, which were also shown (Lockridge, 1971) to be hydrolysed by the enzyme in a Ca^{2+}-dependent manner.

By using β-phenylpropionylthiocholine (R–CO–SR′) and similar substrates, we have developed a direct continuous method for measuring the production of the thio-alcohol (i.e. thiocholine; R′SH) in the enzymic reactions with human plasma γ-glutamyltransferase (Curtis *et al.*, 1974*a*). The kinetic analysis revealed that the pathway of catalysis with this enzyme (E–SH), apparently involving the participation of a critical cysteine residue of the protein

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(Curtis *et al.*, 1973, 1974*b*; Holbrook *et al.*, 1973; Chung *et al.*, 1974), proceeded through an acyl-enzyme (R–CO–S–E) intermediate. Deacylation could take place by hydrolysis and also by aminolysis if an amine substrate (H_2NR'') was present:

$$R\text{–}CO\text{–}SR' + E\text{–}SH \rightleftharpoons (R\text{–}CO\text{–}SR' \cdot E\text{–}SH) \rightarrow R\text{–}CO\text{–}S\text{–}E + R'SH \quad (1)$$

$$R\text{–}CO\text{–}S\text{–}E + H_2O \rightarrow R\text{–}CO_2H + E\text{–}SH \quad (2)$$

$$R\text{–}CO\text{–}S\text{–}E + H_2NR'' \rightleftharpoons (R\text{–}CO\text{–}S\text{–}E \cdot H_2NR'') \rightarrow R\text{–}CONHR'' + E\text{–}SH \quad (3)$$

Among the labelled amine substrates examined, *N*-(5-aminopentyl)-5-dimethylaminonaphthalene-1-sulphonamide or dansylcadaverine (Lorand *et al.*, 1968) proved to be of particular value on account of its fluorescence. With an apparent Michaelis constant of about 1×10^{-4}M, this compound has already been used successfully for such diverse biological investigations as the quantitative study of hereditary deficiency of the enzyme precursor in plasma (Lorand *et al.*, 1970), titration of the order and extent of reactivity of the cross-linking sites in the γ and α chains of fibrin (Lorand *et al.*, 1972*b*), and activity staining of the enzyme on disc-gel electrophoretograms (Lorand *et al.*, 1974).

Amide formation in the reaction between dansylcadaverine and β-phenylpropionylthiocholine could be followed directly through fluorescence measurements by monitoring the continuous extraction of the water-insoluble coupling product into *n*-heptane, layered above the enzymic mixture. In the present paper advantage was taken of this novel method for analysing the steady-state kinetics of both human plasma and guinea-pig liver γ-glutamyltransferase. This work was presented in part at the Federation Meetings, Atlantic City, N.J., April 1973 (Chen *et al.*, 1973).

Materials and Methods

The zymogenic form of human plasma γ-glutamyltransferase and bovine thrombin were isolated by procedures given in our earlier reports (Curtis *et al.*, 1974*a*,*b*). Human platelet and guinea-pig liver γ-glutamyltransferase were prepared by published methods (Schwartz *et al.*, 1971; Folk & Cole, 1966). Concentration of the potential enzymic centres in the purified plasma enzyme was measured by titration with iodoacetamide in the presence of 0.1M-$CaCl_2$ at pH7.5, after limited proteolysis with thrombin (Curtis *et al.*, 1973, 1974*b*). The functional normality of liver γ-glutamyltransferase was determined by the kinetic burst method, with *p*-nitrophenyl trimethylacetate as titrant (Folk *et al.*, 1967). Activation of plasma and platelet zymogens was accomplished by incubating 1mg of these proteins with 3.5 NIH units of thrombin in 1ml of 50mM-Tris–acetate buffer, pH7.5, at 18°C for 20min just before kinetic studies. The thrombin-modified zymogens were stored on ice for the duration of the experiments. Protein concentration of the platelet zymogen was computed by assuming $E^{1\%}_{1cm} = 13.8$ at 280nm (Schwartz *et al.*, 1973).

The concentration of dansylated compounds was measured by absorbancy at 327nm, by using an ε value of 4.67×10^6 litre·mol^{-1}·cm^{-1} (Deranleau & Neurath, 1966). Fluorescence measurements were carried out with an Aminco–Bowman Ratio Spectrophotofluorimeter, in a thermostatically controlled (25°C) cell holder and a circular cuvette of 10mm diameter. In the procedure for kinetic measurements (see the Results section) analytical-grade *n*-heptane (Fisher H-340) was used as the organic phase.

Organic synthesis

β-Phenylpropionic acid, β-phenylpropionic anhydride and thiacadaverine [bis-(2-aminoethyl) sulphide] were purchased from K & K (Hollywood, Calif., 90028, U.S.A.); 5-[*methyl*-^{14}C]dimethylaminonaphthalene-2-sulphonyl chloride ([*methyl*-^{14}C]-dansyl chloride) was obtained from Schwarz–Mann (Van Nuys, Calif. 91401, U.S.A.). Non-radioactive dansyl chloride was purchased from Pierce Chemical Co. (Rockford, Ill. 61105, U.S.A.). 2-Dimethylaminoethanethiol hydrochloride, isobutyl chloroformate and 1,5-diaminopentane were obtained from Aldrich Chemical Co. (Fairfield, N.J., U.S.A.). All solvents used in synthesis were of analytical grade.

Melting points were determined with a Unitron micro hot-stage apparatus and are uncorrected. The mass spectrogram was recorded with a CEC 21-104 apparatus at 70eV. Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer in 10ml of scintillation fluid [toluene (2.4litres)–Triton X-100 (1.2litres) – 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.24g)–2,5-diphenyloxazole (0.6g)].

2-*Dimethylaminoethanethiol* (*base*)

A solution of 2-dimethylaminoethanethiol hydrochloride (14.2g; 0.1mol) in water (10ml) was neutralized by the addition of an aqueous solution (10ml) of NaOH (4.0g; 0.1mol). NaCl (about 2g) was added and the mixture was extracted with diethyl ether (4×20ml). The combined ether extracts were filtered and the solvent was distilled off at atmospheric pressure. The residual oil was distilled at 56–59°C at 8397.9Pa (63mmHg); literature value 40°C at 1599.6Pa (12mmHg) (Fakstorp & Christiansen, 1954).

β-Phenylpropionylthiocholine iodide (*Lorand et al.*, 1972*c*)

To a solution of β-phenylpropionic acid (2.4g; 16mmol) in *p*-dioxan (200ml) were added triethylamine (1.5g; 2.1ml; 15mmol) and isobutyl chloroformate (2.1g: 2.0ml; 15mmol). After 1h at room temperature, the mixture was filtered and 2-dimethylaminoethanethiol (in the base form; 1.4g; 1.5ml; 13mmol) was added to the filtrate. After another 1h at room temperature, methyl iodide (2.8g; 1.2ml; 20mmol) was added and the mixture was left overnight. The precipitated product was then collected by filtration, carefully washed with anhydrous diethyl ether and recrystallized from ethanol. Yield: 3.5g (71%); m.p. 205–206°C (Found: C, 44.04; H, 6.16; N, 3.63. Calc. for $C_{14}H_{22}NOSI$; C, 44.43; H, 5.85; N, 3.69%).

N-(5-Aminopentyl)-5-dimethylaminonaphthalene-1-sulphonamide (*Lorand et al.*, 1968)

This was prepared in one step by the method of Nilsson *et al.* (1971). The crude crystalline base (about 5g) was dissolved in ethanol (150ml), then anhydrous diethyl ether (500ml) was admixed, followed by the addition of a saturated solution of fumaric acid in ethanol until no further precipitate was formed. The precipitated product was collected by filtration, washed with anhydrous diethyl ether and recrystallized from ethanol–anhydrous diethyl ether. M.p. 148–150°C (Found: C, 55.50; H, 6.49; N, 8.91. Calc. for $C_{17}H_{25}N_3O_2S$, $C_4H_4O_4$; C, 55.85; H, 6.47; N, 9.31%).

N-(5-Amino-3-thiapentyl)-5-dimethylaminonaphthalene-1-sulphonamide, ½ *fumarate* (*dansylthiacadaverine*, ½ *fumarate*)

This was synthesized as described by Ljunggren *et al.* (1974).

N-(5-Aminopentyl)-5-[methyl-^{14}C]dimethylaminonaphthalene-1-sulphonamide fumarate ([*methyl-^{14}C*]-*dansylcadaverine fumarate*

A solution of dansyl chloride (100mg; 371μmol) containing 200μCi of 5-[*methyl-*^{14}C]dimethylaminonaphthalene-1-sulphonyl chloride in chloroform (12ml) was added dropwise over a period of 30min to a mixture of 1,5-diaminopentane (87μl; 742μmol) and triethylamine (52μl; 370μmol) in chloroform (2ml). After another 15min at room temperature with stirring, the mixture was washed consecutively with water (15ml), 50%-satd. aq. $NaHCO_3$ (3×15ml), water (15ml) and satd. aq. NaCl (15ml). The organic phase was dried over Na_2SO_4 and filtered. Then anhydrous diethyl ether (10ml) was added to the filtrate followed by a dropwise addition of a satd. solution of fumaric acid in ethanol (2ml). After 30min, the precipitate was collected, washed carefully with anhydrous diethyl ether and recrystallized from ethanol–anhydrous diethyl ether. Yield: 76.8mg; specific radioactivity 0.675μCi/μmol (170μmol = 115μCi) (57%). M.p. 148–150°C. T.l.c. on silica gel (SIL-G, Macherey Nagel & Co., Duren, Germany) developed with conc. NH_3 (sp.gr. 0.880)–ethanol–diethyl ether (2:5:18, by vol.), followed by radioautography, showed a single radioactive spot with R_F 0.55, corresponding to authentic dansylcadaverine fumarate.

N-[5-(β-Phenylpropionylamino)pentyl]-5-dimethylaminonaphthalene-1-sulphonamide or *N-(β-phenylpropionyl)dansylcadaverine*

This was prepared by mixing dansylcadaverine (in the base form; 0.67g; 2mmol) with β-phenylpropionic anhydride (0.56g; 2mmol) in methylene chloride (35ml) at room temperature for 20h. The solution was then washed with 0.1M-HCl (2×30ml), water (2×50ml) and satd. aq. $NaHCO_3$ (2×50ml). After drying over Na_2SO_4, the solvent was evaporated *in vacuo*, yielding 0.90g (96%) of a yellow fluorescent oil with R_F 0.5 on silica-gel (SIL-G) t.l.c. developed with acetone–anhydrous diethyl ether (1:4, v/v). The mass spectrum showed the mass peak at m/e = 467 (calculated mol.wt. 467).

Results and Discussion

A schematic drawing of the procedure for the continuous monitoring of amide formation in the reaction of the positively charged β-phenylpropionylthiocholine with dansylcadaverine is shown in Fig. 1. At pH7.5, chosen for the enzymic experiments, dansylcadaverine exists predominantly in the protonated form and would preferentially partition into the water phase. The uncharged water-insoluble coupling product, however, is being continuously extracted into the upper *n*-heptane phase in the cuvette where it can be readily determined by direct fluorescence measurements at an excitation wavelength of 340nm and an emission wavelength of 460nm. The situation may be illustrated as in Scheme 1. Ca^{2+} ions, necessary for the actions of the enzymes investigated, were added last to the water phase to initiate the reactions. This procedure made it possible to check if any non-specific Ca^{2+}-independent amide formation occurred. In all instances, however, the rates for the non-specific coupling reactions were found to be negligible in terms of corrections for enzymic velocities.

In order to be able to convert relative fluorescence readings into actual quantities of the amide-coupling product, *N*-(β-phenylpropionyl)dansylcadaverine,

an enzymic reaction catalysed by liver γ-glutamyltransferase was carried out between β-phenylpropionylthiocholine and [*methyl*-^{14}C]dansylcadaverine. As the reaction progressed, small samples were withdrawn from the *n*-heptane phase at various fluorescence readings for radioactivity counting; this made it possible to establish an accurate calibration curve (Fig. 2). Some samples were also taken for t.l.c. [on silica gel, in acetone–ether (1:4, v/v)], and it was verified that the fluorescent radioactive compound appearing in the *n*-heptane phase during the enzymic reaction corresponded to *N*-(β-phenylpropionyl)-dansylcadaverine independently synthesized by the procedure described in the Materials and Methods section.

A linear correspondence could be demonstrated between the steady-state formation of the fluorescent product and the number of functional enzymic sites present. This correlation is illustrated for plasma γ-glutamyltransferase in Fig. 3, where the values on the abscissa were obtained from the specific titration of the active centres on the protein by iodoacetamide (Curtis *et al.*, 1973, 1974*b*). It might be pointed out that, for everyday laboratory use, the data in Fig. 3 are of some practical significance in the sense that it is now possible to estimate the number of functional sites of plasma γ-glutamyltransferase preparations simply on the basis of a single steady-state velocity measurement. Besides thrombin (Kézdy *et al.*, 1965), plasma γ-glutamyltransferase is the second enzyme operating in blood coagulation for which such a ready means of standardization could be developed.

Reactions of plasma γ-glutamyltransferase, initiated by the addition of Ca^{2+} ions to the thrombin-activated zymogenic form, show an appreciable lag phase before the steady-state turnover of substrates, which under certain conditions may last for several minutes (Lorand *et al.*, 1974; Curtis *et al.*, 1974*a*). Duration of the lag phase depends on the concentration of Ca^{2+} ions and was shown to be related to the unmasking of the active-centre cysteine residue in the catalytic a′-subunit of the protein (Curtis *et al.*, 1973, 1974*b*) by a process that is linked to a dissociation from the heterologous b subunit (Lorand *et al.*, 1974; Chung *et al.*, 1974).

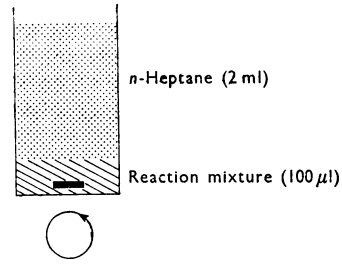
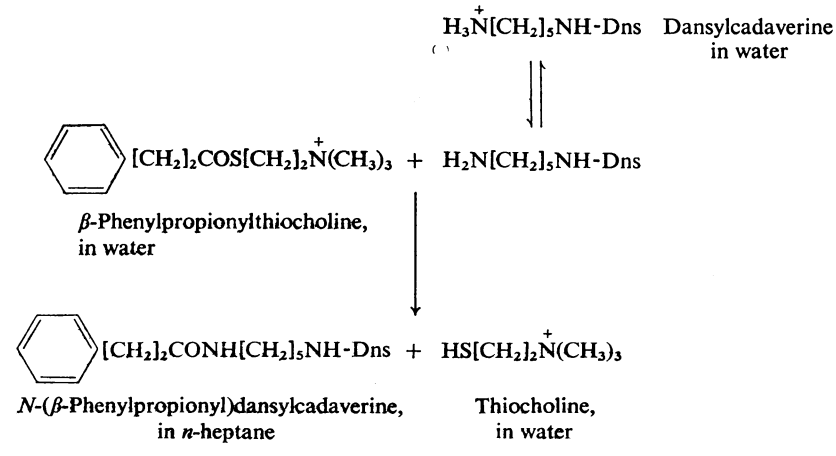


Fig. 1. *Schematic diagram of the two-phase system for measuring amide formation in the reaction of β-phenylpropionylthiocholine iodide with dansylcadaverine*

These two substrates, as well as the enzyme (or zymogen) and Ca^{2+} ions, comprised the lower aqueous phase, which was approximately 0.1 ml in volume and contained 50 mM-Tris–acetate buffer, pH 7.5. Extraction of water-insoluble amide coupling product, *N*-(β-phenylpropionyl)dansylcadaverine, into the *n*-heptane phase was facilitated by stirring the reaction mixture with a magnetic bar at 500 rev. min. Slit and positioning of the cuvette were adjusted such that only the fluorescence (excitation wavelength = 340 nm; emission wavelength = 460 nm) appearing in the upper phase was recorded. All reactions were carried out at 25°C.



Scheme 1. *γ-Glutamyltransferase-catalysed aminolysis of β-phenylpropionylthiocholine with dansylcadaverine as amine donor*

By contrast, progress curves with guinea-pig liver γ-glutamyltransferase gave much shorter lag periods, as shown in Fig. 4 for the reactions of 0.36mM-dansylcadaverine with various concentrations (0.33–1.05mM) of β-phenylpropionylthiocholine. With regard to the lag phase, we reported (Lorand *et al.*, 1974) that after thrombin activation the platelet γ-glutamyltransferase behaved very similarly to liver γ-glutamyltransferase. Addition of the pure *b* subunit, isolated from the plasma zymogen, imposed a kinetic lag on the thrombin-activated platelet enzyme without having any effect on liver γ-glutamyltransferase. As seen in Fig. 5, significant differences among the three γ-glutamyltransferases are also apparent with regard to their Ca^{2+} requirements for catalysing the reaction between dansylcadaverine and β-phenylpropionylthiocholine.

The general versatility of the biphasic fluorescent technique is illustrated by the fact that the procedure is applicable for evaluating inhibition by non-fluorescent amines. Histamine, a known substrate for guinea-pig liver γ-glutamyltransferase (Clarke *et al.*, 1959), inhibits the formation of the fluorescent

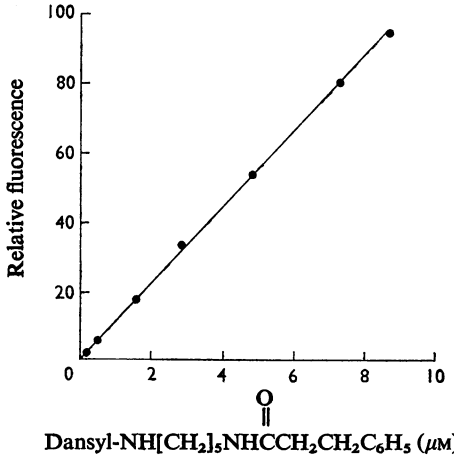


Fig. 2. *Calibration curve for the relationship between relative fluorescence in the n-heptane and actual concentration of amide-coupling product*

The aqueous phase contained 2mM-β-phenylpropionylthiocholine iodide, 1mM-[^{14}C]dansylcadaverine (specific radioactivity 0.675mCi/mmol), 58μM-guinea-pig liver γ-glutamyltransferase and 10mM-$CaCl_2$, added last. The amide product, both fluorescent and radioactive, was continuously extracted into the 2ml *n*-heptane phase from which, at times corresponding to various fluorescent readings (ordinate; arbitrary units), 50μl portions were withdrawn and immediately mixed with 10ml of scintillation fluid for radioactivity counting. After correction for counting efficiency, concentrations on the abscissa were computed on the basis of 2.2×10^6d.p.m. corresponding to 1μCi of radioactive substance.

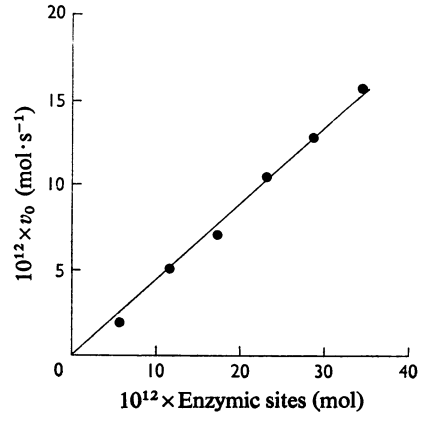


Fig. 3. *Steady-state velocity of amide formation with various amounts of human plasma γ-glutamyltransferase*

The aqueous phase comprised 0.93mM-β-phenylpropionylthiocholine iodide, 0.57mM-dansylcadaverine, various amounts of the thrombin-activated zymogen and finally 70mM-$CaCl_2$. The number of enzymic sites (abscissa) generated from the activated zymogen on exposure to Ca^{2+} was measured by titration with iodoacetamide as described by Curtis *et al.* (1973, 1974*b*). Steady-state velocities of amide formation (ordinate) were obtained from linear portions of progress curves of fluorescence in the organic phase.

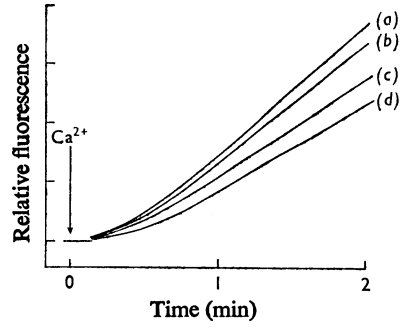


Fig. 4. *Progress curves for guinea-pig liver γ-glutamyltransferase-catalysed reactions of β-phenylpropionylthiocholine with dansylcadaverine*

The aqueous phase contained 0.36mM-dansylcadaverine, various concentrations of β-phenylpropionylthiocholine iodide (1.05, 0.66, 0.44 and 0.33mM respectively for curves *a–d*), 0.77μM-guinea-pig liver γ-glutamyltransferase and 10mM-$CaCl_2$. Note the brief lag period before the steady-state formation of fluorescent product. Progress curves would be quite similar with platelet γ-glutamyltransferase, but the human plasma γ-glutamyltransferase would give rise to a considerably longer lag (Lorand *et al.*, 1974).

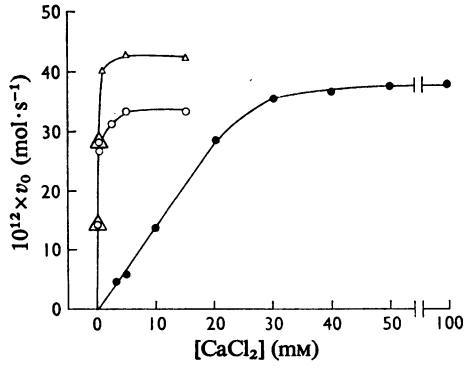


Fig. 5. *Apparent Ca^{2+} ion requirement for three related transamidating enzymes*

The aqueous reaction mixtures contained 8.2mM-β-phenylpropionylthiocholine iodide, 1.6mM-dansylcadaverine, 50μg of platelet γ-glutamyltransferase/ml (△), or 0.50μM-guinea-pig liver γ-glutamyltransferase (○), or 0.32μM-human plasma γ-glutamyltransferase (●), and $CaCl_2$ at the concentrations shown on the abscissa, added last to initiate amide formation (ordinate), which was measured from the steady-state increase of fluorescence in the *n*-heptane phase.

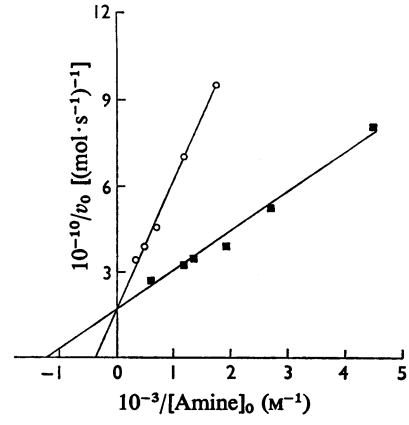


Fig. 6. *Comparison of dansylcadaverine and dansylthiacadaverine in plasma γ-glutamyltransferase-catalysed reactions with β-phenylpropionylthiocholine*

The aqueous mixtures contained 1.86mM-β-phenylpropionylthiocholine iodide, various concentrations of dansylcadaverine (0.29–1.44mM; ○) or dansylthiacadaverine (0.11–0.83mM; ■), 0.28μM-human plasma γ-glutamyltransferase and, finally, 70mM-$CaCl_2$. Reciprocal steady-state velocities of formation of the fluorescent coupling products (ordinate) are plotted against the reciprocal of initial concentrations ([amine]$_0$; abscissa) of the amine substrates. Apparent K_m values are 1.4×10^{-3}M for dansylcadaverine and 0.4×10^{-3}M for dansylthiacadaverine. From the intercepts on the ordinate $k_{cat.} = 2.1 s^{-1}$ was calculated.

coupling product in a competitive manner. From the slope ratios of the Lineweaver–Burk plots a $K_{i,app.}$ of about 6×10^{-4}M was obtained for histamine, which is in good agreement with measurements in another system (Lorand *et al.*, 1972*a*).

The other obvious advantage of the analytical system lies in being able to perform kinetic measurements with fluorescent amines other than dansylcadaverine. Though the latter is an extremely useful compound, recent work in this laboratory indicated (Curtis *et al.*, 1974*b*) that some of its analogues were even better substrates. As seen in Fig. 6, a comparison of dansylcadaverine with dansylthiacadaverine shows that, in the plasma γ-glutamyltransferase-catalysed reactions with β-phenylpropionylthiocholine, both amines elicit identical maximum velocities for forming their respective amide products. The apparent K_m, however, is about three times more favourable for the thiacadaverine derivative. Similar conclusions were drawn also from the measurement of thiocholine product formation in the presence of the two amine substrates (Curtis *et al.*, 1974*a*).

Initial steady-state velocities of amide formation were examined at four different concentrations of dansylcadaverine for the plasma and liver γ-glutamyltransferase-catalysed reactions, with β-phenylpropionylthiocholine as the varied substrate. With reference to the plasma enzyme, this was the first time that such a relatively complete kinetic analysis could be performed with a synthetic substrate pair. Such studies have been reported (Chung *et al.*, 1970) for liver γ-glutamyltransferase, but only in conjunction with the rather poor ($K_{m,app.}$ 50mM) *N*-benzyloxycarbonylglutaminylglycine or the very labile *p*-nitrophenyl acetate substrates, reacting with amines (e.g. [^{14}C]alanine ethyl ester) of low specificity, and by having to measure the formation of amide product by sampling of the reaction mixtures at various times.

Lineweaver–Burk plots, relating to variations of initial concentrations of both the ester and amine substrates, are given in Fig. 7 for plasma γ-glutamyltransferase. Similar quality results, in which the lines intercept on the abscissa, were obtained with liver γ-glutamyltransferase. The limiting maximum velocities (V) for infinite concentrations of the two substrates (represented by the solid triangle on the ordinate of Fig. 7) were obtained graphically by extrapolation of the secondary plots in Fig. 8. These extrapolated values permitted calculation of catalytic rate constants ($k_{cat.} = V/E_0$, where E_0 = concentration of potential enzymic sites) of $1.8 s^{-1}$ and $0.9 s^{-1}$ for plasma and liver γ-glutamyltransferase respectively. This is the first instance that a kinetic comparison between the two enzymes was at all possible, with catalytic-centre activity being normalized on the basis of functional active sites rather than per mg of

enzyme protein. A comparison of specific activities of the latter type, for methylamine substitution by the two enzymes into the acetylated β chain of oxidized insulin, has been published (Chung & Folk, 1972). Although in that work guinea-pig liver γ-glutamyltransferase gave the higher specific rate (by a factor of nearly 2), in the present study the plasma γ-glutamyltransferase appears to be slightly favoured. The similarity of molar catalytic-centre activity ($k_{cat.}$) for the two enzymes is all the more impressive if one considers that active sites (E_0) in each were titrated by entirely different procedures, employing the Ca^{2+}-dependent alkylation of active-centre thiol groups for the plasma enzyme (Curtis *et al.*, 1973, 1974*b*) and measuring kinetic burst with *p*-nitrophenyl trimethylacetate for the liver enzyme (Folk *et al.*, 1967).

The set of intersecting lines (Fig. 7) for amide (R–CO–NHR′) formation in the enzymic reactions of β-phenylpropionylthiocholine (R–CO–SR′) with dansylcadaverine (H_2NR'') is in accord, of course, with the formulation of a branched pathway, as was postulated from an examination of the kinetics of thiocholine (R′SH) production (Curtis *et al.*, 1974*a*). The situation may be illustrated as in Scheme 2.

By using the steady-state approximation and assuming that $k_{-1} \gg k_{+2}$ and $k_{-4} \gg k_{+5}$, the rate law for the velocity (v) of amide formation is given by the expression:

$$v = \frac{\left(\frac{k_{+2}+k_{+5}}{k_{+2}\cdot k_{+5}}\right)E_0}{1+\left(\frac{k_{+5}}{k_{+2}+k_{+5}}\cdot\frac{K_a}{a}\right)+\left(\frac{k_{+2}+k_{+3}}{k_{+2}+k_{+5}}\cdot\frac{K_b}{b}\right)+\left(\frac{k_{+3}}{k_{+2}+k_{+5}}\cdot\frac{K_a\cdot K_b}{ab}\right)}$$

where E_0 is the number of enzymic sites, a and b represent the concentrations of the ester and amine substrates respectively, $K_a = k_{-1}/k_{+1}$, $K_b = k_{-4}/k_{+4}$, k_{+2} is the rate constant for acylation of the enzyme; k_{+3} and k_{+5} are the hydrolytic and aminolytic rate constants for deacylation. It may be seen that, at infinite concentrations of the two substrates (a, $b \to \infty$), the limiting maximum velocity is given by:

$$V = \frac{k_{+2}\cdot k_{+5}}{k_{+2}+k_{+5}}\cdot E_0 \qquad \left(\text{i.e. } k_{cat.} = \frac{k_{+2}\cdot k_{+5}}{k_{+2}+k_{+5}}\right)$$

Such plots for the reactions catalysed by the two enzymes (see, e.g., Fig. 7) form a family of lines intersecting at a single point on the abscissa. This behaviour would be consistent with the above function if $k_{+2} \ll k_{+5}$ and k_{+3}, i.e. if the rate-limiting step was the formation of the acyl-enzyme (R–CO–S–E) intermediate. Under these circumstances the point of intersection corresponds to $-1/K_a$. A comparison of the

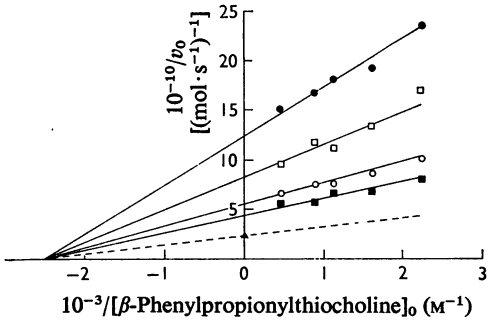


Fig. 7. *Kinetic analysis of the steady-state production of amide with plasma glutamyltransferase*

The aqueous-phase mixtures comprised various initial concentrations of β-phenylpropionylthiocholine iodide (0.44–2.1 mM; reciprocals shown on the abscissa), initial concentrations of dansylcadaverine fixed at 0.20 (●), 0.28 (□), 0.57 (○) and 0.97 mM (■) respectively, 0.26 μM-plasma γ-glutamyltransferase and, finally, 70 mM-$CaCl_2$. Reciprocal steady-state velocities for the appearance of the fluorescent coupling product in the organic phase are given on the ordinate. ▲, Value for limiting maximum velocity of amide production obtained graphically by extrapolation of a secondary plot (see Fig. 8) to infinite concentration of amine substrate. Thus ---- would indicate reaction velocities for infinite concentration of dansylcadaverine at various initial concentrations of β-phenylpropionylthiocholine.

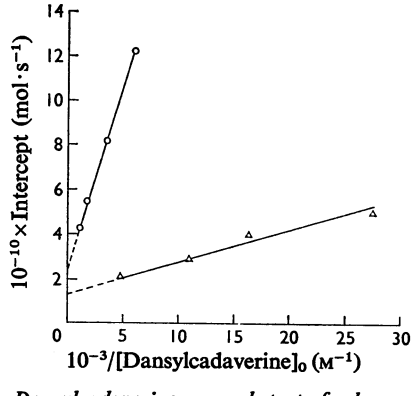
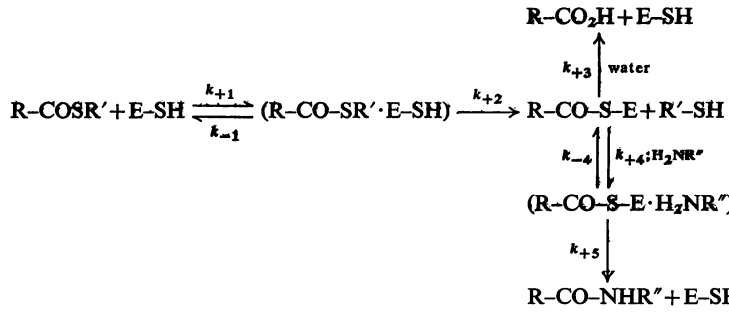


Fig. 8. *Dansylcadaverine as a substrate for human plasma* (○) *and guinea-pig liver γ-glutamyltransferase* (△)

Secondary plots for the intercepts on the vertical axis (see Fig. 7) as a function of the reciprocal of initial concentration of dansylcadaverine.



Scheme 2. *Postulated pathway for the γ-glutamyltransferase-catalysed hydrolysis and aminolysis of thiol esters*

plasma and liver γ-glutamyltransferase showed that both enzymes have identical specificities for β-phenylpropionylthiocholine, with $K_a = 4 \times 10^{-4}$ M. However, other studies (Curtis *et al.*, 1974*b*), in which the free thiocholine was measured in the presence and the absence of an added amine, suggest that $k_{+2} > k_{+3}$ i.e. the hydrolysis of β-phenylpropionylthiocholine is deacylation rate limiting. In this case when $1/v = 0$ and $k_{+2} \ll k_{+5}$ the above function simplifies to:

$$\frac{1}{a} = -\frac{1}{K_a}\left[\frac{1+\left(\frac{k_{+2}+k_{+3}}{k_{+5}}\cdot\frac{K_b}{b}\right)}{1+\left(\frac{k_{+3}}{k_{+5}}\cdot\frac{K_b}{b}\right)}\right]$$

and $1/a = -1/K_a$ only if the expressions

$$\left(\frac{k_{+2}+k_{+3}}{k_{+5}}\cdot\frac{K_b}{b}\right) \quad \text{and} \quad \left(\frac{k_{+5}}{k_{+3}}\cdot\frac{K_b}{b}\right)$$

are small with respect to 1.

It is also evident from the rate equation of amide formation that a secondary plot (as in Fig. 8) of the intercepts (at $a = \infty$) of the primary curves in Fig. 7 as a function of $1/b$ would yield a straight line with a slope of:

$$\left(\frac{k_{+2}+k_{+3}}{k_{+2}+k_{+5}}\right)\cdot\frac{K_b}{V}$$

In case $k_{+2} \ll k_{+5}$, this simplified to:

$$\left(\frac{k_{+2}+k_{+3}}{k_{+5}}\right)\cdot\frac{K_b}{V}$$

and since it has been suggested (Curtis *et al.*, 1974*b*) that k_{+2} is not affected by the presence of an added amine, then the reciprocal of the latter expression may be taken as an index of the efficiency of utilization of the amine substrate by the enzyme. A comparison of the slopes of the lines for the two enzymes in Fig. 8 shows that, by this criterion, liver γ-glutamyltransferase is approximately one order of magnitude more efficient than plasma γ-glutamyltransferase in utilizing dansylcadaverine.

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