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Determination of the Operational Molarity of Solutions of Bovine α-Chymotrypsin, Trypsin, Thrombin and Factor X*a* by Spectrofluorimetric Titration

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Several esters of 4-methylumbelliferone and 2-naphthol were synthesized and examined as possible spectrofluorimetric titrants for bovine α-chymotrypsin, trypsin, thrombin, Factor X*a* and for subtilisin Novo. 4-Methylumbelliferyl *p*-guanidinobenzoate hydrochloride (MUGB) is a satisfactory titrant for α- and β-trypsin, thrombin and Factor X*a* and 4-methylumbelliferyl *p*-(*NNN*-trimethylammonium)cinnamate (MUTMAC) is a good titrant for α-chymotrypsin. The amount of enzyme used for spectrofluorimetric titration is 0.02–3.00nmol and the amount of 4-methylumbelliferone liberated is independent of the concentration of titrant and stoicheiometrically equal to the amount of enzyme used. Results obtained with MUGB and MUTMAC have been checked by spectrophotometric titration with *p'*-nitrophenyl *p*-guanidinobenzoate hydrochloride and *p*-nitrophenyl N^2-acetyl-N^1-benzylcarbazate respectively. *p*-Nitrophenyl N^2-acetyl-N^1-(9-anthrylmethyl)carbazate has been synthesized; it did not react with α-chymotrypsin. A satisfactory spectrofluorimetric titrant for subtilisin Novo was not discovered.

Several spectrophotometric methods for determining the operational molarity of solutions of chymotrypsin, trypsin and thrombin have been developed here and elsewhere (Kézdy *et al.*, 1965; Bender *et al.*, 1966; Chase & Shaw, 1967, 1969; Elmore & Smyth, 1968*a*,*b*; Baird & Elmore, 1968; Heidema & Kaiser, 1968; Knowles & Preston, 1968; Tanizawa *et al.*, 1968, 1970; Latif & Kaiser, 1969; Vaughan & Westheimer, 1969). No methods, however, have been described for the spectrophotometric titration of Factor X*a*. Unfortunately, spectrophotometric titrations with *p*-nitrophenyl esters require at least 1–2nmol of enzyme even if a narrow cuvette with a light-path length of 4–5cm is used. Consequently, we have synthesized a number of quasi-substrates in which a 4-methylumbelliferyl or 2-naphthyl ester group is present with a view to devising suitable spectrofluorimetric titration methods for the serine proteases. The present paper reports methods for the spectrofluorimetric titration of bovine α- and β-trypsin, thrombin, Factor X*a* and α-chymotrypsin. A preliminary account of this work has been published (Roberts *et al.*, 1971).

Materials

Trypsin (twice-crystallized, salt-free) was purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) and separated chromatographically into the α- and β-forms as described by Schroeder & Shaw (1968). Further samples of α- and β-trypsin were kindly donated by Dr. E. Shaw. α-Chymotrypsin (thrice-crystallized, salt-free) was purchased from Worthington Biochemical Corp. or from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and was purified by the method of Nakagawa & Bender (1970). Bovine thrombin and Factor X*a* were prepared from blood plasma by the methods of Magnusson (1965*a*,*b*) and Esnouf *et al.* (1973) respectively. Subtilisin Novo was purified by the method of Ottesen & Spector (1960).

The following compounds were synthesized as described in the literature: *p*-nitro-L-phenylalanine monohydrate (Bergel & Stock, 1954); *p*-(*NNN*-trimethylammonium)cinnamic acid iodide (Knowles & Preston, 1968); α-*N*-benzoyl-L-arginine ethyl ester and α-*N*-methyl-α-*N*-toluene-*p*-sulphonyl-L-lysine 2-naphthyl ester hydrobromide (Elmore & Smyth, 1968*a*); *p*-nitrophenyl N^2-acetyl-N^1-benzylcarbazate (Elmore & Smyth, 1968*b*); *p*-guanidinobenzoic acid hydrochloride (Beyerman & Bontekoe, 1953); *p'*-nitrophenyl *p*-guanidinobenzoate hydrochloride (Chase & Shaw, 1967).

S-(3-*Aminopropyl*)-*N*-*toluene*-*p*-*sulphonyl*-L-*cysteine hydrochloride*

A solution of *N*-toluene-*p*-sulphonyl-L-cysteine (11g) and 3-bromopropylamine hydrochloride (10g) in 2M-NaOH (50ml) was left at room temperature

until a test for thiol group was negative. The pH was adjusted to 7 and the precipitate was collected, washed with water, and dissolved in 2M-HCl. The solution was extracted with ethyl acetate to remove traces of *NN'*-bis(toluene-*p*-sulphonyl)-L-cystine. The aqueous solution was evaporated to dryness under reduced pressure and the *amino acid hydrochloride* (71%) was recrystallized from ethanol–diethyl ether when it had m.p. 228–230°C (decomp.) (Found: C, 42.5; H, 6.0; N, 7.7. $C_{13}H_{21}ClN_2O_4S_2$ requires C, 42.3; H, 5.7; N, 7.6%).

S-(3-t-Butyloxycarbonylaminopropyl)-N-toluene-p-sulphonyl-L-cysteine

To a suspension of *S*-(3-aminopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine (6.6g) in water (24ml) and dioxan (30ml) was added magnesium oxide (1.04g) and t-butylazidoformate (4.4ml) and the mixture was stirred for 6h at 40°C. The dioxan was removed under reduced pressure and the solution was acidified to pH2.5 with ice-cold 1M-H_2SO_4 and extracted with ethyl acetate. The ethyl acetate solution was extracted with saturated $KHCO_3$ solution which was then acidified with ice-cold 1M-H_2SO_4 and extracted with ethyl acetate. The ethyl acetate solution was washed with water and dried ($MgSO_4$). Addition of light petroleum (b.p. 60–80°C) caused the *product* (72%) to crystallize. After recrystallization from ethyl acetate – light petroleum, it had m.p. 104–106°C (Found: C, 50.0; H, 6.3; N, 6.7. $C_{18}H_{28}N_2O_6S_2$ requires C, 50.0; H, 6.5; N, 6.5%).

S-(3-t-Butyloxycarbonylaminopropyl)-N-toluene-p-sulphonyl-L-cysteine 2-naphthyl ester

S-(3-t-Butyloxycarbonylaminopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine (2.04g) and bis-(2-naphthyl) sulphite (1.21g) were dissolved in freshly distilled and dried pyridine (10ml) and the solution was left overnight. After removal of the pyridine under reduced pressure, the residual oil was dissolved in ethyl acetate and the solution was washed successively at 0°C with 1M-HCl, saturated $NaHCO_3$ solution, water and then dried ($MgSO_4$). Addition of light petroleum (b.p. 60–80°C) caused the *ester* (69%) to crystallize. After recrystallization from ethyl acetate–light petroleum, it had m.p. 76–78°C (Found: C, 60.1; H, 6.1; N, 5.1. $C_{28}H_{34}N_2O_6S_2$ requires C, 60.2; H, 6.1; N, 5.0%).

S-(3-Aminopropyl)-N-toluene-p-sulphonyl-L-cysteine 2-naphthyl ester hydrogen sulphate

S-(3-t-Butyloxycarbonylaminopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine 2-naphthyl ester (1g) was dissolved in a mixture of ethyl acetate (10ml) and ether (10ml). A mixture of ether (20ml) and conc. H_2SO_4 (2.5ml) was added and the solution was stored at 0°C. The *product* (72%) was collected, washed with ether and recrystallized from t-butanol–ether when it had m.p. 164–166°C (Found: C, 49.4; H, 5.2; N, 5.2. $C_{23}H_{28}N_2O_8S_3$ requires C, 49.6; H, 5.1; N, 5.0%).

N^2-Acetyl-N^1-(9-anthrylmethylene)hydrazine

A solution of 9-anthraldehyde (5.0g) and acethydrazide (1.8g) in ethanol was shaken vigorously for a few minutes and then cooled. The *product* (5.8g) was collected, recrystallized from ethyl acetate–light petroleum (b.p. 60–80°C) when it had m.p. 244–246°C (Found: C, 77.8; H, 5.6; N, 10.5. $C_{17}H_{14}N_2O$ requires C, 77.8; H, 5.4; N, 10.7%).

N^2-Acetyl-N^1-(9-anthrylmethyl)hydrazine

A solution of N^2-acetyl-N^1-(9-anthrylmethylene) hydrazine (2.6g) in dry tetrahydrofuran (200ml) was saturated with diborane at −5°C and left overnight at room temperature. A mixture of ethanol (50ml) and 10M-HCl (2ml) was added and the solution was evaporated to dryness under reduced pressure. A solution of the residue in ethyl acetate was filtered, washed with water, dried ($MgSO_4$) and concentrated under reduced pressure; crystallization was induced by addition of light petroleum (b.p. 60–80°C). After recrystallization from dichloromethane–light petroleum (b.p. 60–80°C), the *product* (1.1g) had m.p. 204°C (Found: C, 78.0; H, 6.3; N, 10.5. $C_{17}H_{16}N_2O$ requires C, 77.3; H, 6.1; N, 10.6%).

p-Nitrophenyl-N^2-acetyl-N^1-(9-anthrylmethyl)-carbazate

A cold solution of *p*-nitrophenyl chloroformate (0.77g) in dry dichloromethane (10ml) was added during 30min to a solution of N^2-acetyl-N^1-(9-anthrylmethyl)hydrazine (1.0g) and triethylamine (0.4g) in dry dichloromethane (15ml) at 0°C. The mixture was left overnight at 4°C, washed with 2M-HCl, water, ice-cold $NaHCO_3$ solution, water and dried ($MgSO_4$). The solution was concentrated under reduced pressure and the *product* (0.8g) was caused to crystallize by addition of light petroleum (b.p. 40–60°C). After recrystallization from ethyl acetate–light petroleum (b.p. 60–80°C), it had m.p. 196°C (Found: C, 66.8; H, 4.5; N, 9.7. $C_{24}H_{19}N_3O_5$ requires C, 67.1; H, 4.5; N, 9.8%).

N-Toluene-p-sulphonyl-L-p-nitrophenylalanine

Toluene-*p*-sulphonylchloride (14g) in diethyl ether (200ml) was stirred with a solution of L-*p*-nitrophenylalanine monohydrate (11.5g) with periodic addition

of 4M-KOH at pH 10.0 (pH-stat). When reaction was complete as judged by alkali uptake and a ninhydrin test (approx. 4h), the ethereal layer was removed and the aqueous phase was extracted twice with ethyl acetate. The aqueous phase was acidified with HCl (pH <2) and extracted with ethyl acetate (3×200ml). The combined extracts were dried ($MgSO_4$), evaporated and treated with light petroleum (b.p. 40–60°C). The *product* (76%), after recrystallization from ethyl acetate – light petroleum (b.p. 40–60°C), had m.p. 187–189°C (Found: C, 52.9; H, 4.3; N, 8.0. $C_{16}H_{16}N_2O_6S$ requires C, 52.7; H, 4.4; N, 7.7%).

N-Methyl-N-toluene-p-sulphonyl-L-nitrophenylalanine

Dimethyl sulphate (7.3g) was added in small portions during 1h to a stirred solution of *N*-toluene-*p*-sulphonyl-L-*p*-nitrophenylalanine (9.6g) in water (450ml) with the pH maintained at 12.0 (pH-stat) by addition of 4M-KOH. The solution was stirred at pH 12.0 for a further 3h, treated with solid KOH (56g), and stirred for 2 days. The solution was acidified with HCl to pH 1 and extracted with dichloromethane (3×150ml). The combined extracts were dried ($MgSO_4$), evaporated to dryness, and the residual *product* (87%) was crystallized from ethyl acetate – light petroleum (b.p. 60–80°C). After recrystallization from dichloromethane – light petroleum (b.p. 60–80°C), it had m.p. 180–182°C (Found: C, 53.8; H, 5.1. $C_{17}H_{18}N_2O_6S$ requires C, 54.0; H, 4.8%).

α-N-Methyl-α-N-toluene-p-sulphonyl-L-p-aminophenylalanine hydrochloride

N-Methyl-*N*-toluene-*p*-sulphonyl-L-*p*-nitrophenylalanine (8.7g) was hydrogenated at atmospheric pressure over 5% palladium–barium sulphate (870 mg) in methanol (200ml) containing 10M-HCl (6ml) for 4h. The solution was filtered, evaporated to small bulk, and the *product* (82%) was caused to crystallize by addition of diethyl ether. The m.p., which was dependent on the rate of heating, was 244–247°C (decomp.) and was unchanged after recrystallization from ethanol–diethyl ether (Found: C, 53.1; H, 5.6. $C_{17}H_{21}ClN_2O_4S$ requires C, 53.0; H, 5.5%).

α-N-Methyl-α-N-toluene-p-sulphonyl-L-p-guanidinophenylalanine toluene-p-sulphonate

α-*N*-Methyl-α-*N*-toluene-*p*-sulphonyl-L-*p*-aminophenylalanine hydrochloride (3.85g) and 3,5-dimethyl-1-guanidinopyrazole nitrate (2.01g) were dissolved in water (200ml) and the pH was adjusted to 7 with NaOH solution. The solution was heated under reflux for 24h, cooled, adjusted to pH 8, filtered and the filtrate (A) was retained. The brown residue was extracted with hot 2M-HCl (200ml), the extract was cooled, adjusted to pH 8 and the product was collected. Filtrate A was heated under reflux with more 3,5-dimethyl-1-guanidinopyrazole nitrate (2.01g) for 24h, more product was isolated as before and the whole procedure was done for a third time. The combined crops (37%) of the guanidino acid, which did not melt up to 320°C, were neutralized in methanolic solution with toluene-*p*-sulphonic acid and the *salt* (1.75g) crystallized on addition of diethyl ether. A sample which was recrystallized twice from a very dilute aqueous solution of toluene-*p*-sulphonic acid and washed liberally with dry diethyl ether, had m.p. 220–221°C (Found: C, 53.5; H, 5.4; N, 9.9. $C_{25}H_{30}N_4O_7S_2$ requires C, 53.4; H, 5.4; N, 10.0%). Attempts to guanidinate α-*N*-methyl-α-*N*-toluene-*p*-sulphonyl-L-*p*-aminophenylalanine hydrochloride by heating it with cyanamide under reflux in ethanol, propan-1-ol or water were unsuccessful. The progress of guanidination reactions and the purity of products were checked by high-voltage electrophoresis on Whatman No. 1 paper at pH 5.2, 3.6 and 1.9 in the following buffers: (1) pyridine (20g)–acetic acid (9.5g)–water to 1 litre; (2) pyridine–acetic acid–water (1:10:189, by vol.); (3) formic acid–acetic acid–water (2.5:7.8:89.7, by vol.). Electrophoretograms were run for 30min at 4–5kV and guanidino compounds were detected by spraying with an alkaline solution of phenanthraquinone (Yamada & Itano, 1966).

α-N-Methyl-α-N-toluene-p-sulphonyl-L-p-guanidinophenylalanine 4-methylumbelliferyl ester toluene-p-sulphonate

α-*N*-Methyl-α-*N*-toluene-*p*-sulphonyl-L-*p*-guanidinophenylalanine toluene-*p*-sulphonate (113mg), 4-methylumbelliferone (42mg) and *NN'*-dicyclohexylcarbodi-imide (49mg) were stirred overnight in a mixture of *N*-methylpyrrolid-2-one (1.5ml) and pyridine (1.5ml). The mixture was filtered and the crude *product* crystallized after careful addition of diethyl ether to the filtrate. After recrystallization from *NN*-dimethylformamide–diethyl ether and then from methanol–diethyl ether, the product (32%) had m.p. 155–157°C. For analysis, a sample was extracted with a small volume of ice-cold 0.01M-HCl and recrystallized once from methanol – diethyl ether and thrice from dry 2-methylbutan-2-ol when it had m.p. 154–156°C (Found: C, 58.3; H, 5.3; N, 7.8. $C_{35}H_{36}N_4O_9S_2$ requires C, 58.3; H, 5.0; N, 7.8%).

p-(NNN-Trimethylammonium)cinnamic acid chloride

p-(*NNN*-Trimethylammonium)cinnamic acid iodide (Knowles & Preston, 1968) (2.4g) in water was applied to a column of Bio-Rad AG1-X4 (100–200 mesh) in the hydroxide form. The column was

eluted with methanol and the eluate was concentrated under reduced pressure, acidified with 2M-HCl and evaporated to dryness under reduced pressure. The resulting *salt* (1.4g) was recrystallized from methanol–diethyl ether when it had m.p. 198–200°C and was free of iodide ions (Found: C, 60.0; H, 6.8; N, 6.2. $C_{12}H_{16}ClNO_2$ requires C, 59.6; H, 6.7; N, 5.8%). All batches of this compound prepared subsequently had m.p. 234–237°C and were nearly insoluble in chloroform–dichloromethane (1:1, v/v). In contrast, the form with the lower m.p. was quite soluble in this solvent. Both forms, however, had the same infrared and proton-magnetic-resonance spectra and the same elemental composition.

4-Methylumbelliferyl p-(NNN-trimethylammonium)-cinnamate chloride (MUTMAC)

p - (*NNN* - Trimethylammonium)cinnamic acid chloride (0.8g) was dissolved in freshly distilled thionyl chloride (25ml) and left for 12h. The solution was evaporated to dryness under reduced pressure at room temperature and the residue was dissolved in dry acetonitrile (20ml). A solution of 4-methylumbelliferone (0.59g) and tri-*n*-butylamine (0.62g) in dry acetonitrile (20ml) was added in four batches (5ml) with mixing at 0°C. Precipitation began immediately. When the reaction appeared to be complete, dry diethyl ether (50ml) was added. The precipitate (1.1g) was collected, slurried with water (10ml) on the sintered-glass funnel, sucked dry and then washed successively with acetone and diethyl ether. A portion of the *ester hydrochloride* (0.2g) was dissolved by shaking with water (5ml) and caused to crystallize by the addition of acetone (50ml) and sufficient diethyl ether to saturate the solution; it then had m.p. 204–206°C (Found: C, 63.5; H, 5.3; Cl, 8.7; N, 3.2. $C_{22}H_{22}ClNO_4,H_2O$ requires C, 63.2; H, 5.8; Cl, 8.5; N, 3.4%). One sample of the ester hydrochloride which proved to be identical with the above product was synthesized from the specimen of *p*-(*NNN*-trimethylammonium)cinnamic acid chloride having m.p. 198–200°C and 4-methylumbelliferone by the mixed anhydride procedure with ethyl chloroformate in a mixture of chloroform and dichloromethane. This method, however, could not be used with the specimens of *p*-(*NNN*-trimethylammonium) cinnamic acid chloride having m.p. 234–237°C because the solubility in chloroform–dichloromethane was too low for reaction to proceed to completion.

4-Methylumbelliferyl p-guanidinobenzoate hydrochloride (MUGB)

p-Guanidinobenzoic acid hydrochloride (1.07g), 4-methylumbelliferone (0.88g) and *NN'*-dicyclohexylcarbodi-imide (1.08g) were dissolved in a mixture of pyridine (7.5ml) and *NN*-dimethylformamide (7.5ml) and the solution was left overnight. *NN'*-Dicyclohexylurea was collected and washed with a mixture of pyridine (5ml) and *NN*-dimethylformamide (5ml). The combined filtrate and washings were evaporated to dryness under reduced pressure and 0.1M-HCl (25ml) was added. The slurry was extracted with ethyl acetate (3×25ml) and the phases were separated by centrifugation. The *product* (59%) was collected by filtration from the aqueous phase. After drying under vacuum over P_2O_5, it was recrystallized from acetic acid–diethyl ether and then from 2-methylbutan-2-ol–diethyl ether when it had m.p. 219–221°C (Found: C, 55.6; H, 4.4; N, 11.1; Cl, 9.3. $C_{18}H_{16}ClN_3O_4,H_2O$ requires C, 55.2; H, 4.6; N, 10.7; Cl, 9.0%).

N-Toluene-p-sulphonyl-L-p-aminophenylalanine hydrochloride

N-Toluene-*p*-sulphonyl-L-*p*-nitrophenylalanine (10g) in ethyl acetate (50ml) and ethanol (50ml) was hydrogenated in the presence of 10M-HCl (5ml) and 10% palladium on charcoal (0.75g). After reduction, ethanol (50ml) was added and the solution was warmed to 60°C and filtered hot. Addition of diethyl ether to the cooled solution caused the *product* (69%) to crystallize. It was recrystallized from ethanol–diethyl ether and had m.p. 217–219°C (decomp.) (Found: C, 52.1; H, 5.5; N, 7.7. $C_{16}H_{19}ClN_2O_4S$ requires C, 51.8; H, 5.2; N, 7.6%). Attempted guanidination of this compound with cyanamide in methanol afforded only 10–20% of the required product.

N-Toluene-p-sulphonyl-L-p-nitrophenylalanine methyl ester

N-Toluene-*p*-sulphonyl-L-*p*-nitrophenylalanine (5g) was esterified by azeotropic distillation with methanol–benzene (1:1, v/v) in the presence of toluene-*p*-sulphonic acid (0.5g). After evaporation under reduced pressure, the residue was dissolved in ethyl acetate (100ml) and washed successively with saturated $NaHCO_3$ solution and water and then dried ($MgSO_4$). Addition of light petroleum (b.p. 60–80°C) caused the *product* (72%) to crystallize. After recrystallization from ethyl acetate–light petroleum (b.p. 60–80°C), it had m.p. 120–122°C (Found: C, 53.8; H, 4.6; N, 7.5. $C_{17}H_{18}N_2O_6S$ requires C, 53.9; H, 4.8; N, 7.4%).

N-Toluene-p-sulphonyl-L-p-aminophenylalanine methyl ester hydrochloride

N-Toluene-*p*-sulphonyl-L-*p*-nitrophenylalanine methyl ester (4.0g) in methanol (50ml) was hydrogenated in the presence of 10% palladium on $CaCO_3$

(0.2g). After reduction, the solution was warmed to 60°C, filtered hot and 10M-HCl (2ml) was added. Addition of diethyl ether to the cooled solution caused the product (79%) to crystallize. After recrystallization from methanol–diethyl ether, it had m.p. 218–220°C (Found: C, 53.3; H, 5.4; N, 7.5. $C_{17}H_{21}ClN_2O_4S$ requires C, 53.1; H, 5.5; N, 7.3%). This compound was also prepared by esterification of *N*-toluene-*p*-sulphonyl-L-*p*-aminophenylalanine hydrochloride with methanol saturated with dry HCl. Guanidination with cyanamide in methanol containing HCl gave 60–70% of the desired product but isolation in a pure state was not possible.

N-Toluene-p-sulphonyl-L-p-thioureidophenylalanine

N-Toluene-*p*-sulphonyl-L-*p*-aminophenylalanine (3.7g) and ammonium thiocyanate (1.2g) were dissolved in 1M-HCl (13ml) and the solution was evaporated to dryness in an evaporating basin on a steam bath. The residue was dissolved in 2.5M-NaOH and the solution was filtered and acidified. The *product* (46%), after recrystallization from ethyl acetate–light petroleum (b.p. 60–80°C), had m.p. 174–176°C (Found: C, 52.2; H, 5.0; N, 10.5. $C_{17}H_{19}N_3O_4S_2$ requires C, 51.9; H, 4.9; N, 10.7%).

N-Toluene-p-sulphonyl-L-p-guanidinophenylalanine toluene-p-sulphonate

A solution of *N*-toluene-*p*-sulphonyl-L-*p*-thioureidophenylalanine (1.0g) and methyl iodide (0.4g) in ethanol (15ml) was heated under reflux for 2h. The solution was evaporated to dryness, treated with aq. 25% (w/v) NH_3 (20ml) and heated for 3h on a water bath. The solution was acidified to pH5 with acetic acid and stored in a refrigerator. The crystalline guanidino acid was collected after 24h, washed with ice-cold water, suspended in hot ethanol and treated with toluene-*p*-sulphonic acid until dissolution was complete. After cooling and addition of diethyl ether, the *product* (48%) crystallized. After recrystallization from ethanol–diethyl ether and then from t-butanol–diethyl ether, it had m.p. 159–161°C (Found: C, 50.9; H, 5.6; N, 9.9. $C_{24}H_{28}N_4O_7S_2,H_2O$ requires C, 50.9; H, 5.3; N, 9.9%). Attempted synthesis of the 4-methylumbelliferyl ester by using *NN'*-dicyclohexylcarbodi-imide failed to give a pure product.

Methods

Preparation of solutions

The following buffers were used in spectrofluorimetric and spectrophotometric assays: 0.05M- or 0.1M-sodium barbiturate containing enough HCl to give pH8.30; 0.05M-Na_2HPO_4 – 0.05M-NaH_2PO_4, pH7.70; 8.695mM-KH_2PO_4 – 30.43mM-Na_2HPO_4, pH7.41 (National Bureau of Standards, standard buffer); 0.05M-Na_2HPO_4–0.05M-NaH_2PO_4, pH7.00; 0.025M-KH_2PO_4–0.025M-Na_2HPO_4, pH6.86 (National Bureau of Standards standard buffer); 0.05M-sodium cacodylate containing enough HCl to give pH6.30; 0.05M-Na_2HPO_4 – 0.05M-NaH_2PO_4, pH6.30; 0.05M-sodium acetate – 0.05M-acetic acid, pH5.32; 0.05M-sodium acetate – 0.05M-acetic acid, pH4.7; 0.05M-sodium acetate – 0.05M-acetic acid, pH4.01. All buffers were filtered through sintered-glass funnels before use.

The stock solutions of enzymes used were: 10–20μM-trypsin in 1mM-HCl; 10–20μM-thrombin in 0.15M-NaCl; 10–20μM-α-chymotrypsin in 1mM-HCl; 2–8μM-bovine Factor X*a* in 0.01M-Na_2HPO_4–0.01M-NaH_2PO_4, pH7.7, containing 0.15M-NaCl; 10–20μM-subtilisin Novo in 0.02M-$CaCl_2$. All the above solutions were prepared freshly as required except that of Factor X*a* which was isolated in that form and stored at –20°C in small vials.

Stock solutions of MUGB (10mM) and α-*N*-methyl-α-*N*-toluene-*p*-sulphonyl-L-*p*-guanidinophenylalanine 4-methylumbelliferyl ester toluene-*p*-sulphonate (1mM) were prepared in *N*-methylpyrrolid-2-one. These were diluted to 0.1–0.2mM with 1mM-HCl before use and the dilute solutions could be stored frozen for several weeks without serious deterioration. Solutions (0.05–0.2mM) of MUTMAC were made up in water and could also be stored frozen for several weeks.

Fluorescence measurements

The intensity of fluorescence due to 4-methylumbelliferone or 2-naphthol was measured with a Zeiss ZFM4C spectrofluorimeter. The excitation wavelength was isolated from the output of a 450W xenon arc lamp by means of a M4QIII prism monochromator and passed into the sample cuvette held in a ZFM4 fluorescence attachment maintained at 25°C. The sample cuvettes contained either 1ml or 3ml of solution. The emission wavelength was isolated by a M2O grating monochromator and its intensity was measured by a PMQ II galvanometer connected to a 'Servoscribe' recorder. Quinine (0.25mg/l or 1.0mg/l) in 0.1M-H_2SO_4 or a Zeiss fluorescence standard (F53) were used as fluorescence intensity standards and a linear relationship between fluorescence intensity and 4-methylumbelliferone concentration was demonstrated over the range 0.02–1.0μM, although normally concentrations were not allowed to exceed 0.2μM. Excitation was at 365nm at pH values above 7.0 and at 330nm at pH7.0 and below. Emission was observed at 445nm. With 2-naphthol, excitation was at 325nm at pH4.7 and at 330nm at pH7.4. Emission was observed at 410nm at both pH values.

Spectrophotometric determination of the absolute molarity of solutions of trypsin and α-chymotrypsin

Solutions of trypsin were titrated with *p'*-nitrophenyl *p*-guanidinobenzoate hydrochloride by a slight modification of the method of Chase & Shaw (1967). Stock solutions (0.02M) of reagent were prepared in *N*-methylpyrrolid-2-one. The latter solvent was preferred to *NN*-dimethylformamide since the stock solution was stable for several months at 0°C. Solutions of α-chymotrypsin were titrated with *p*-nitrophenyl N^2-acetyl-N^1-benzylcarbazate by the method of Elmore & Smyth (1968*b*).

Spectrofluorimetric determination of the absolute molarity of solutions of proteases

(*a*) *Normal method.* Substrate solution (10–250μl) and enzyme solution (10–100μl) were successively added to the requisite buffer (3ml) in a cuvette maintained at 25°C. The change in fluorescence intensity with time was recorded and compared with that of a control containing no enzyme. The measurements of fluorescence of 4-methylumbelliferone were made briefly and intermittently since the latter undergoes photolytic decomposition. Two of the substrates, MUGB and MUTMAC, also underwent photolytic decomposition, but only when the excitation wavelength was 330nm (i.e. when working at or below pH7.0). Errors due to photolysis were decreased to an acceptable level by using higher concentrations of substrate and enzyme than usual and by taking very brief measurements of fluorescence with the more concentrated quinine solution as standard. α-*N*-Methyl-α-*N*-toluene-*p*-sulphonyl-L-*p*-guanidinophenylalanine 4-methylumbelliferyl ester toluene-*p*-sulphonate was appreciably hydrolysed in the absence of catalyst above pH7.0; the reaction appeared to be catalysed by cacodylate but not phosphate buffer at the same pH. The volumes of substrate and enzyme solutions used were chosen to give substrate concentration/enzyme concentration ratios in the range 1.3–80. Typically, and particularly in the first examination of a given enzyme–substrate system, the enzyme and substrate concentrations in the cuvette were 0.1–0.2μM and 1.0μM respectively.

(*b*) *Concentrated incubation method.* For the titration of enzymes that reacted inconveniently slowly with substrate, a modified technique was used. Reaction was allowed to proceed to completion in a very small volume of buffer at relatively high concentrations of enzyme and substrate and then the solution was diluted with a large volume of buffer for fluorescence measurements. Typically, 10μM-enzyme (20μl), 0.2mM-titrant (10μl) and 0.1M-sodium barbiturate buffer, pH8.3 (20μl), were carefully mixed in a small tube. The tube was stoppered and kept at room temperature for 3min (trypsin) to 60min (Factor X*a*). Buffer (1–3ml) was then added and the fluorescence intensity was measured in the usual way. Controls that lacked enzyme were set up and treated in the same way. With care, the method gave reproducible results.

Results

Titration of trypsin

One compound, MUGB, proved to be an excellent titrant for both α- and β-trypsin. When the normal method was used to titrate β-trypsin at pH8.3, the 'burst' was found to be nearly complete within the time required for mixing and instrumental response and it was completely over within 5s. The half-life was probably less than 1s and the second-order rate constant is probably greater than $10^6 M^{-1} s^{-1}$. Four titrations of a solution of β-trypsin with MUGB gave a mean value of 11.8±0.3μM for the enzyme concentration. Titration of the same solution with *p'*-nitrophenyl *p*-guanidinobenzoate hydrochloride gave a value of 11.5μM. The reaction of MUGB with α-trypsin was much slower than with β-trypsin and the 'burst' was complete after about 15min with the normal method at pH8.3 (Fig. 1). Accurate kinetic studies of the reaction have not yet been performed, but the second-order rate constant is probably at least two orders of magnitude lower than for the corresponding reaction with β-trypsin. Six titrations of a solution of α-trypsin with MUGB gave a mean value of 8.36±0.20μM for the enzyme concentration. Titration of the same solution with *p'*-nitro-

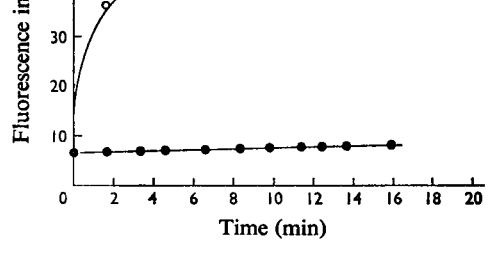

Fig. 1. *Time-course of the reaction between MUGB and α-trypsin*

The increase in fluorescence intensity with time was determined for a mixture of α-trypsin solution (50μl), 0.1M-sodium barbiturate buffer, pH8.3 (3.0ml), and 0.1mM-MUGB (25μl). o, Complete mixture; ●, α-trypsin omitted.

phenyl *p*-guanidinobenzoate hydrochloride gave a value of 8.26μM. Because of the considerable difference in the rates of reaction of MUGB with α- and β-trypsin, it is possible to titrate mixtures of the two enzymes. The concentrated incubation method for enzyme titration with MUGB gave results that did not differ significantly from those obtained by the normal method.

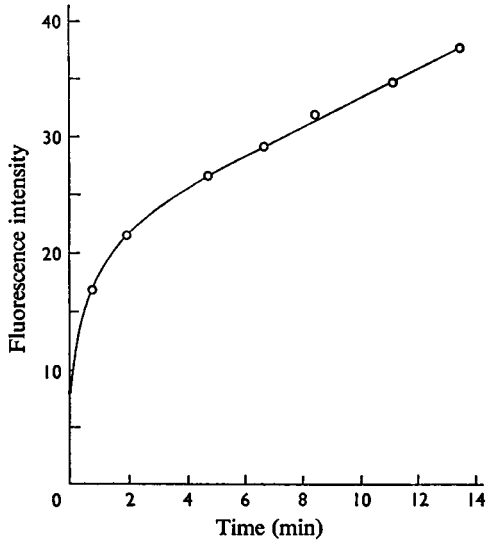

Fig. 2. *Time-course of the reaction between MUGB and thrombin*

The increase in fluorescence intensity with time was determined for a mixture of thrombin solution (30μl), 0.05M-sodium barbiturate buffer, pH8.3 (3.0ml), and 0.2mM-MUGB (30μl).

MUTMAC at pH7.4, α-*N*-methyl-α-*N*-toluene-*p*-sulphonyl-L-*p*-guanidinophenylalanine 4-methylumbelliferyl ester toluene-*p*-sulphonate at pH4.0, α-*N*-methyl-α-*N*-toluene-*p*-sulphonyl-L-lysine 2-naphthyl ester hydrobromide and *S*-(3-aminopropyl)-*N*-toluene-*p*-sulphonyl-L-cysteine 2-naphthyl ester hydrogen sulphate at pH4.7 and 7.41 in the normal method all gave 'bursts' with trypsin solutions that were much lower than expected. Since there was appreciable turnover in each case, it is clear that the requirements that $k_2 \gg k_3$ and hence $[S]_0 \gg K_m$ were not met.

Titration of thrombin

p'-Nitrophenyl *p*-guanidinobenzoate hydrochloride is a titrant for bovine thrombin (Baird & Elmore, 1968; Chase & Shaw, 1969). Not surprisingly, therefore, we found that MUGB is also a suitable titrant. Acylation was complete within about 5min at pH8.3 with the normal method (Fig. 2) but appreciable turnover occurred. Nevertheless, the size of the 'burst' obtained by extrapolation gave an accurate value for the molarity of thrombin. The results of a set of molarity determinations are listed in Table 1. The mean value for the molarity of the stock solution of thrombin is 10.5$\pm$0.4μM with MUGB as titrant. Three determinations with *p'*-nitrophenyl *p*-guanidinobenzoate hydrochloride gave a mean value of 10.7$\pm$0.5μM for the same solution. Examination of the results in Table 1 shows that (*a*) the method is insensitive to a fivefold variation in concentration of

Table 1. *Titration of thrombin with MUGB*

The normal method was used with 0.2mM-MUGB in mM-HCl. The buffer was 0.05M-sodium barbiturate, pH8.30.

Volume of MUGB solution (μl)	Volume of thrombin solution (μl)	Final volume (ml)	'Burst'	Concentration of stock thrombin solution (μM)
30	30	3.06	37.0	10.4
30	50	3.08	60.6	10.3
30	30	3.06	38.2	10.7
10	30	3.04	34.6	9.76
30	30	3.06	36.5	10.3
50	30	3.08	37.4	10.6
10	30	3.04	37.2	10.5
30	30	3.08	36.4	10.3
50	30	3.08	37.6	10.6
30	30	3.06	37.1	10.4
30	30	3.06	37.3	10.5
30	10	3.04	13.5	11.4
				Mean = 10.5$\pm$0.4μM

MUGB and (*b*) the size of the 'burst' is proportional to the concentration of enzyme. Since the rate of turnover of MUGB in the presence of thrombin is considerably greater than with either α- or β-trypsin, it was decided to determine if the turnover occurred at the active site or if it was the result of non-specific catalysis. When thrombin was pretreated with 7-amino-1-chloro-3-toluene-*p*-sulphonamidobutan-2-one, the inhibited enzyme did not give a burst in the presence of MUGB and the linear increase in fluorescence did not exceed that of a control containing MUGB at the same concentration, pH and temperature (cf. Chase & Shaw, 1969). Clearly, the turnover of MUGB involves the active site of thrombin.

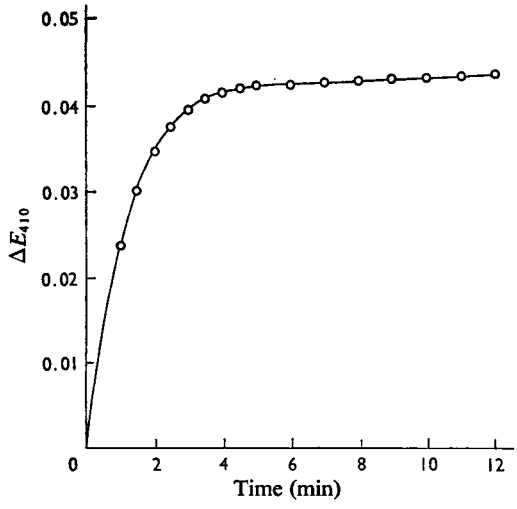

Fig. 3. *Time-course of the reaction between p'-nitrophenyl p-guanidinobenzoate and Factor Xa*

The increase in E_{410} with time was determined for a solution which was 2.51 μM with respect to Factor X*a* and 33.2 μM with respect to *p'*-nitrophenyl *p*-guanidinobenzoate in 0.1 M-sodium barbiturate buffer, pH 8.3.

When α-*N*-methyl-α-*N*-toluene-*p*-sulphonyl-L-*p*-guanidinophenylalanine 4-methylumbelliferyl ester toluene-*p*-sulphonate was allowed to react with thrombin at pH 7.0, a burst was observed that was much smaller than that observed with MUGB and there was extensive turnover. No more favourable behaviour was observed at lower pH values; both acylation and deacylation appeared to be decelerated to about the same extent until, at pH 4.0, reaction was almost undetectable. No reaction was detected when MUTMAC was added to a solution of thrombin at pH 8.3.

Titration of Factor Xa

No method for determining the operational molarity of solutions of Factor X*a* has been described hitherto and consequently we decided to examine initially the possible use of *p'*-nitrophenyl *p*-guanidinobenzoate hydrochloride as a spectrophotometric titrant. Factor X*a* liberated *p*-nitrophenol from *p'*-nitrophenyl *p*-guanidinobenzoate hydrochloride at pH 8.3 in amounts that were proportional to the amount of enzyme present and turnover was negligible (Fig. 3). Acylation was complete after about 5 min. When the concentration of titrant was much greater than that of Factor X*a*, pseudo-first-order kinetics were observed. Although accurate kinetic measurements have not yet been made, the second-order rate constant is approximately 390 $M^{-1}s^{-1}$ at pH 8.3 and 25°C. This rate constant is at least four orders of magnitude lower than the rate constant for the reaction with β-trypsin and about forty times lower than for the reaction with thrombin.

Titrations with *p'*-nitrophenyl *p*-guanidinobenzoate hydrochloride require rather large amounts of

Table 2. *Titration of Factor Xa with MUGB: proportionality of amount of liberated 4-methylumbelliferone to enzyme concentration*

The concentrated incubation method was used with Factor X*a* solution (v μl), 0.1 M-sodium barbiturate buffer, pH 8.3 (20 μl), 0.01 M-sodium phosphate buffer containing 0.15 M-NaCl, pH 7.7 (20 − v μl) and 0.2 mM-MUGB in mM-HCl (10 μl). Controls (v = 0) were prepared similarly. The solutions were incubated at room temperature for 60 min and diluted with 0.05 M-sodium barbiturate buffer, pH 8.3 (1.00 ml) before measurement of the fluorescence.

Volume of enzyme solution added (v μl)	Concentration of 4-methylumbelliferone in cuvette (c nM)	Concentration of enzyme solution ($1.05c/v$ μM)
5	16.5	3.47
10	37.1	3.90
15	53.6	3.75
20	70.1	3.68

enzyme and, in the case of Factor X*a*, it was convenient to establish a secondary assay for esterase activity. With a stock solution of Factor X*a* that had been standardized with *p'*-nitrophenyl *p*-guanidinobenzoate hydrochloride, the rate constant for the steady-state hydrolysis of 5mM-α-*N*-benzoyl-L-arginine ethyl ester hydrochloride was $5.6\pm0.3\,s^{-1}$ at pH8.0 and 25°C in 0.1M-NaCl. Rate assays based on this value were then used to determine the operational molarity of solutions of Factor X*a* in experiments that were designed to assess the accuracy of spectrofluorimetric titrations of the same enzyme solutions.

Although scarcely detectable by the standard spectrofluorimetric titration method (e.g. with 2μM-MUGB and 0.05μM-Factor X*a*) at pH8.3, the rate of this reaction was sufficiently increased under the conditions used for the concentrated incubation method at pH8.3 (e.g. with 40μM-MUGB and 0.8μM-Factor X*a*) that measurements of the operational molarity of the enzyme solution were possible. In the concentrated solution, acylation was complete in less than 20min, although the incubation time used was usually 60min. No turnover was detected. Concordant results were obtained when the operational molarity of a solution of Factor X*a* was determined by the steady-state assay with α-*N*-benzoyl-L-arginine ethyl ester hydrochloride (4.10μM) and by the spectrofluorimetric assay with MUGB (4.13 and 4.20μM). The amount of 4-methylumbelliferone liberated was proportional to the amount of enzyme used (Table 2) and independent of the concentration of MUGB (Table 3). The second-order rate constant for the reaction of MUGB with Factor X*a* at pH8.3 was approximately $50\,M^{-1}s^{-1}$. α-*N*-Methyl-α-*N*-toluene-*p*-sulphonyl-L-*p*-guanidinophenylalanine 4-methylumbelliferyl ester toluene-*p*-sulphonate reacted rather slowly with turnover at pH7.0. A very small 'burst' was observed but this could not be equated with the amount of enzyme present. MUTMAC was barely affected by Factor X*a*.

Titration of α-chymotrypsin

When α-chymotrypsin reacted with MUTMAC, acylation was complete in less than 2min at pH values between 7.4 and 8.3 and the rate of deacylation was negligible. A solution of α-chymotrypsin was assayed spectrofluorimetrically with MUTMAC

Table 3. *Titration of Factor Xa with MUGB: independence of amount of liberated 4-methylumbelliferone and amount of MUGB used*

The concentrated solution method was used with Factor X*a* solution (20μl), 0.1M-sodium barbiturate buffer, pH8.3 (15μl), 0.2mM-MUGB in mM-HCl ($x\mu$l) and mM-HCl ($25-x\mu$l). The solutions were incubated at room temperature for 60min and diluted with 0.05M-sodium barbiturate solution, pH8.3 (1.00ml), before measurement of the fluorescence.

Concentration of MUGB in incubation mixture (μM)	Concentration of 4-methylumbelliferone in cuvette (*c*nM)	Concentration of enzyme solution ($1.06c/20\mu$M)
16.7	68.7	3.64
33.3	69.3	3.67
50.0	77.5	4.11
66.7	71.1	3.77

Table 4. *Titration of α-chymotrypsin with MUTMAC: proportionality of amount of liberated 4-methylumbelliferone to enzyme concentration*

The normal method was used with pH7.4 buffer (3.0ml) and 50μM-MUTMAC (50μl) in the cuvette.

Volume of enzyme solution added ($v\mu$l)	Concentration of 4-methylumbelliferone in cuvette (*c*nM)	Concentration of enzyme solution [$c(3.05+0.001v)/v\mu$M]
10	37	11.3
20	78	12.0
30	121	12.4
40	157	12.1
50	198	12.3
60	225	11.7

Table 5. *Titration of α-chymotrypsin with MUTMAC: independence of amount of liberated 4-methylumbelliferone and amount of MUTMAC used*

The normal method was used with pH7.4 buffer (3.0ml) and enzyme solution (30μl) in the cuvette.

Concentration of MUTMAC in cuvette (μM)	Molarity of enzyme solution (μM)
0.164	11.6
0.409	11.6
0.812	12.4
1.600	11.9

and spectrophotometrically with *p*-nitrophenyl N^2-acetyl-N^1-benzylcarbazate; the mean values of the concentration of the enzyme were 12.0±0.5(6)μM and 12.5±0.8(3)μM respectively. The amount of 4-methylumbelliferone liberated was proportional to the enzyme concentration (Table 4) and independent of the concentration of MUTMAC (Table 5) and of pH in the range 6.8–8.3.

When α-*N*-methyl-α-*N*-toluene-*p*-sulphonyl-L-*p*-guanidinophenylalanine 4-methylumbelliferyl ester toluene-*p*-sulphonate reacted with α-chymotrypsin, acylation of the enzyme was followed by turnover at an appreciable rate. Although the difference in rates of acylation and deacylation was sufficient to permit the assay of solutions of chymotrypsin, the results appeared to be slightly lower and less precise than those obtained in assays using MUTMAC or *p*-nitrophenyl N^2-acetyl-N^1-benzylcarbazate. Thus, the molarity of the solution of α-chymotrypsin mentioned in the previous paragraph was found to be 11±1μM (15) when α-*N*-methyl-α-*N*-toluene-*p*-sulphonyl-L-*p*-guanidinophenylalanine 4-methylumbelliferyl ester toluene-*p*-sulphonate was used as titrant. At pH8.3, MUGB was hydrolysed by chymotrypsin with no measurable 'burst'. Finally, no evidence was obtained for any reaction between α-chymotrypsin and *p*-nitrophenyl N^2-acetyl-N^1-(9-anthrylmethyl)-carbazate in acetonitrile–water buffer (1:2, v/v) in the apparent pH range of 5.0–8.3, although the reagent underwent non-enzymic hydrolysis at the higher pH values.

Attempted titration of subtilisin Novo

No reaction between subtilisin and MUGB was detected at pH8.3. The enzyme catalysed a slow hydrolysis of α-*N*-methyl-α-*N*-toluene-*p*-sulphonyl-L-*p*-guanidinophenylalanine 4-methylumbelliferyl ester toluene-*p*-sulphonate with no observable 'burst' at pH7.0. A 'burst' of 4-methylumbelliferone was formed from MUTMAC in presence of subtilisin at pH7.0 and pH6.3. Unfortunately, this was followed by fairly rapid turnover and the size of the 'burst' depended on the substrate concentration.

Discussion

The foregoing experiments have demonstrated that solutions of α- and β-trypsin, thrombin, Factor X*a* and α-chymotrypsin can be titrated spectrofluorimetrically with either MUGB or MUTMAC as appropriate and the accuracy and precision compare favourably with earlier spectrophotometric methods. Unlike the latter, which require about 1nmol of enzyme, the spectrofluorimetric methods can be used with as little as 0.02nmol of enzyme. This increase in sensitivity has enabled us to carry out extensive investigations with Factor X*a* and other enzymes that are not available in quantity (T. Morrison, A. G. Lloyd, R. W. Adams, D. H. Hogg, & D. T. Elmore, unpublished work). The search for new spectrofluorimetric titrants, however, is likely to be more difficult than in the case of spectrophotometric reagents. Since both substrate and enzyme concentrations are low in methods employing spectrofluorimetry, it is more difficult to fulfil the kinetic requirements for a suitable titrant (Bender *et al.*, 1966). In particular, because [S] must be considerably greater than K_m and because [S] is low, the rate constant for the acylation step should be very high and turnover must be negligible ($k_2 \gg k_3 \approx 0$). Where the rate of acylation is inconveniently slow, but $k_2 \gg k_3$, the concentrated incubation method can be used.

In the spectrofluorimetric methods that we have described, the fluorescence of the ester titrant is low and the leaving group is the main fluorophore. An alternative approach involves using a titrant in which the group that acylates the enzyme exhibits a large change in fluorescent behaviour after reaction. In particular, a group that occupies the 'tosyl hole' of chymotrypsin might be expected to show different fluorescent properties after acylation of the enzyme. *p*-Nitrophenyl N^2-acetyl-N^1-(9-anthrylmethyl)carbazate was synthesized as a potential reagent of this type. Unfortunately, it did not react with chymotrypsin, presumably because the molecule is too wide (about 0.73nm) to enter the 'tosyl hole', which is reported to have an irregular flattened shape with dimensions 1.0–1.2nm×0.55–0.65nm×0.35–0.40nm (Steitz *et al.*, 1969).

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