N-ACYLATED PHENYLALANINE *p*-NITROANILIDES: NEW SUBSTRATES FOR CHYMOTRYPSIN

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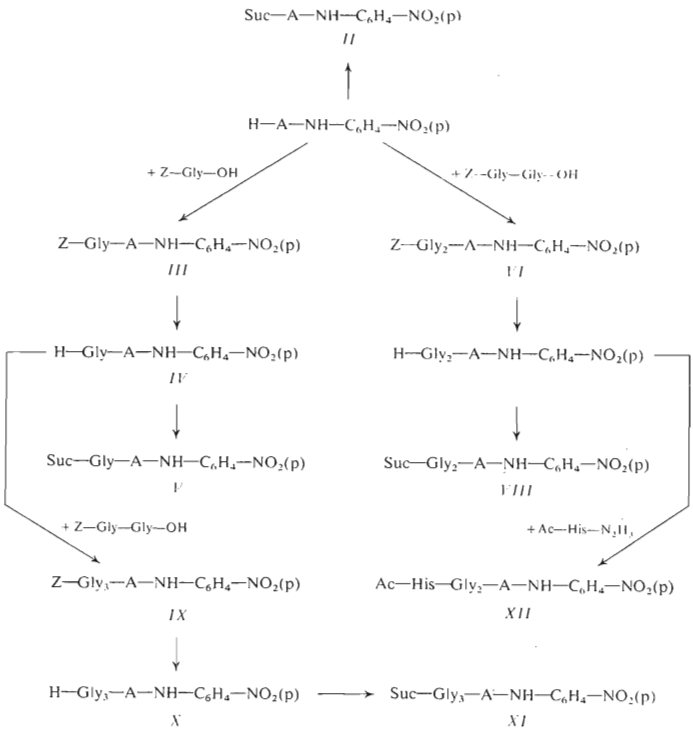
Chromogenic substrates of the following types were synthesized: X-Phenylalanine *p*-nitroanilides, where X = acetyl, hydroxyacetyl, methoxyethoxyacetyl, and 3-carboxy-2-propenoyl, $(glycyl)_n$-phenylalanine *p*-nitroanilides and 3-carboxypropanoyl$(glycyl)_n$-phenylalanine *p*-nitroanilides, where $n = 1,2,3$, $(glycyl)_n$-tyrosine *p*-nitroanilides and 3-carboxypropanoyl$(glycyl)_n$--tyrosine *p*-nitroanilides, where $n = 0,1,2$, and also acetylhistidyl-glycyl-glycyl-phenylalanine *p*-nitroanilide. The constants K_m, k_{cat} and C (*i.e.* k_{cat}/K_m) were estimated for enzymic splitting by chymotrypsin. The greatest k_{cat} values were found for 3-carboxypropanoylglycyl-glycyl--tyrosine *p*-nitroanilide and 3-carboxypropanoyl-glycyl-glycyl-phenylalanine *p*-nitroanilide, 40·5 and 16·9, respectively. The smallest K_m value, $1{\cdot}1 \times 10^{-4}$ (mM) was observed with acetylhistidyl--glycyl-glycyl-phenylalanine *p*-nitroanilide, which exhibited also the greatest C constant, 115000 ($M^{-1}\,s^{-1}$).

Chymotryptic splitting of amide, N-arylamide and peptide bonds is conditioned by the presence of a C-terminal hydrophobic amino acid[1] (primary interaction) and is also affected by neighbouring acyl residues (secondary interactions)[2]. The role of the peptide backbone length and of single amino acid residues vicinal to the splitted bond have been extensively studied using a great variety of substrates. Hydrophobic interactions and hydrogen bonding have been regarded to be decisive in the enzyme–substrate complex formation[3–7]. Electrostatic interactions have not been examined systematically in this context. However, chymotrypic splitting of the 4-carboxybutanoylglycyl-glycyl-phenylalanine β-naphthylamide[8] and 3-carboxypropanoylalanyl--alanyl-phenylalanine (or tyrosine) *p*-nitroanilides[9]* clearly evidenced that the presence of a negatively charged group strongly affects the kinetics of the N-arylamide bond cleavage. The enzyme–substrate binding is affected and also the constants K_m and k_{cat}, *cf.*[10].

For a more detailed study of this effect we synthesized now tailor-made substrates in which the negative charge was located in a variable distance from the splitted bond $P_1 - P_1'$, *cf.*[11]. The N-terminal part of phenylalanine or tyrosine *p*-nitroanilides

* Symbols and nomenclature follow the recommendations published by the IUPAC-IUB Commission on Biochemical Nomenclature for amino acids[12,13]. The chiral amino acids appearing in this paper are of the L-series.

was extended by inserted glycine residues with which we may presume only the hydrogen-bonding participation because of the absence of the hydrophobic side chain. The terminal α-amino groups were acylated by the 3-carboxypropanoyl residue which provided the negatively charged carboxylate function. We synthesized *p*-nitroanilides of the general formulae $(glycyl)_n$-phenylalanine and 3-carboxypropanoyl-$(glycyl)_n$-phenylalanine *p*-nitroanilides, where $n = 1, 2, 3$, and similar substrates



SCHEME 1

In formulae *IIIa*–*XIIa* A = Phe, in formulae *IIIb*–*VIIIb* A = Tyr. Suc for 3-carboxypropanoyl.

containing tyrosine instead of phenylalanine (n = 0, 1, 2 in tyrosine derivatives). We prepared also the acetylhistidyl-glycyl-glycyl-phenylalanine *p*-nitroanilide with imidazole ring in the N-terminal side chain. An additional series of substrates includes N-acylphenylalanine *p*-nitroanilides in which acyl residues are acetyl, hydroxyacetyl, methoxyethoxyacetyl and 3-carboxy-2-propenoyl.

The synthesis of substrates was carried out similarly as in the preceding paper[9] and it is outlined in the Scheme 1. The protected phenylalanine and tyrosine peptides, excluding *XIIa*, were built up using N,N'-dicyclohexylcarbodiimide for coupling.

TABLE I
p-Nitroanilides of Phenylalanine

Compound Yield, %	M.p., °C $[\alpha]_D^{20}$ [a]	Formula (Mol.wt.)	Calculated/Found·		
			% C	% H	% N
IIIa	204—205	$C_{25}H_{24}N_4O_6$	63·01	5·08	11·76
76	+ 8·4°	(476·5)	63·11	5·17	11·87
IVa	176—178	$C_{17}H_{18}N_4O_8$	59·65	5·30	16·37
71	+67·9°	(342·3)	60·23	5·62	16·27
Va	138—140	$C_{21}H_{22}N_4O_7$	56·92	5·00	12·64
77	+21·4°	(443·1)	57·13	5·22	12·47
VIa	188—191	$C_{27}H_{27}N_5O_7$	60·79	5·10	13·13
67	+26·1°	(533·5)	60·87	5·23	12·97
VIIa	95—98	$C_{19}H_{21}N_5O_5$	57·15	5·30	17·54
52	+16·7°	(399·4)	56·45	5·44	17·22
VIIa.HBr	266—268	$C_{19}H_{21}N_5O_5$.HBr	47·52	4·62	14·58
—	—	(480·3)	47·27	4·36	14·16
VIIIa	211—214	$C_{23}H_{25}N_5O_8$	55·23	5·04	14·00
83	+21·3°	(500·17)	55·04	5·10	13·96
IXa	228—229	$C_{29}H_{30}N_6O_8$	58·98	5·17	14·23
61	+28·4°[b]	(590·53)	59·08	5·26	14·51
Xa	216—220	$C_{21}H_{24}N_6O_6.2\,H_2O$	51·22	5·73	17·07
35	+19·4°	(492·4)	51·89	5·46	17·56
XIa	221—224	$C_{25}H_{28}N_6O_9.H_2O$	52·20	5·26	14·61
31	+24·2°[b]	(557·2)	52·51	5·33	15·13
XIIa	265—270	$C_{37}H_{30}N_8O_7.H_2O$	54·36	4·51	18·78
23	+19·7°	(596·6)	54·41	5·20	18·94

[a] In methanol; [b] in N,N-dimethylformamide.

Hydrogen bromide in acetic acid was used for splitting off the benzyloxycarbonyl protecting group. Free α-aminoacyl *p*-nitroanilides were succinylated using succinanhydride in N,N-dimethylformamide at 80°C. *XIIa* was synthesized by the azide method[14] starting from N^{α}-acetylhistidine hydrazide which was prepared in situ by hydrazinolysis from the corresponding methyl ester, *cf.*[15]. The analytical data and yield of compounds of the phenylalanine series are presented in Table I, those of the tyrosine series in Table II. The analogous data of N-acylphenylalanine *p*-nitroanilides are collected in Table III. For acetylation (*Ia*) and 3-carboxy-2-propenoylation (*Ib*) of the phenylalanine *p*-nitroanilide corresponding anhydrides were applied. Synthesis of the hydroxyacetyl derivative *Id* was carried out in two steps: Phenylalanine *p*-nitroanilide was acylated with tert-butyloxyacetic acid[16] using N,N'-dicyclohexylcarbodiimide or activated ester (*p*-nitrophenyl) methods for condensation. From the intermediate *Ic* the protecting group was splitted off by trifluoroacetic acid. The last substrate in this series, *Ie*, was prepared using the methoxyethoxyacetic acid[17] and N,N'-dicyclohexylcarbodiimide or activated esters (*p*-nitrophenyl and 2,4,5-trichlorophenyl) for acylation.

TABLE II
p-Nitroanilides of Tyrosine

Compound Yield, %	M.p. °C $[\alpha]_D^{20}$ [a]	Formula (Mol.wt.)	Calculated/Found		
			% C	% H	% N
IIb	219—223	$C_{19}H_{19}N_3O_7$	56·76	4·76	10·45
62	+16·3°	(402·1)	57·26	4·86	10·28
IIIb	158—160	$C_{25}H_{24}N_4O_7$	60·98	4·91	11·38
65	+31·1 [b]	(492·4)	60·59	4·89	11·45
IVb	157—160	$C_{17}H_{18}N_4O_5$	56·99	5·06	15·64
55	+72·8°	(358·3)	56·38	4·94	15·20
Vb	151—154	$C_{21}H_{22}N_4O_8 \cdot 1/2H_2O$	53·88	4·95	11·97
53	+28·8 [c]	(468·1)	54·33	4·84	11·48
VIb	209—210	$C_{27}H_{27}N_5O_8$	59·02	4·95	12·75
60	+30·1 [b]	(549·5)	58·80	5·40	12·32
VIIb	223—225	$C_{19}H_{21}N_5O_6$	54·94	5·10	16·86
58	+32·0°	(415·4)	55·27	5·28	17·00
VIIIb	189—191	$C_{23}H_{25}N_5O_9$	53·52	4·88	13·57
87	+30·3°	(515·2)	53·88	4·87	13·62

[a] In methanol; [b] in N,N-dimethylformamide; [c] concentration 0·05.

TABLE III

Acylated Phenylalanine *p*-Nitroanilides

Compound Yield, %	M.p., °C $[\alpha]_D^{20a}$	Formula (Mol.wt.)	Calculated/Found % C	% H	% N
Ia	244—246	$C_{17}H_{17}N_3O_4$	62·38	5·24	12·84
88	+97·9°	(327·3)	62·31	5·48	12·87
Ib	171—173	$C_{19}H_{17}N_3O_6$	59·54	4·47	10·96
88	+28·8°	(383·4)	59·62	4·52	11·00
Ic	182—183	$C_{21}H_{25}N_3O_5$	63·16	6·31	10·52
68[b], 82[d]	—	(399·4)	63·19	6·45	10·38
Id	190	$C_{17}H_{17}N_3O_5$	59·47	4·99	12·24
95	+48·1°	(343·3)	59·24	5·01	12·24
Ie	130—132	$C_{20}H_{23}N_3O_6$	59·84	5·77	10·47
63[b], 72[c]	+64·1°	(401·4)	60·00	5·90	10·63

[a] In methanol; [b] carbodiimide method; [c] mixed anhydride method; [d] activated ester.

TABLE IV

Kinetic Constants of Substrates Cleaved by Chymotrypsin

Substrate	K_m, M . 10^{-1}	k_{cat}, s^{-1}	k_{cat}/K_m, M^{-1} s^{-1}
Ia	2·1	0·19	905
Ib	5·5	0·19	345
Id	5·0	0·19	380
Ie	2·7	0·44	1 630
IVa	4·2	0·21	500
Va	5·0	0·81	1 620
VIIa	4·3	1·68	3 907
VIIIa	5·5	16·85	30 636
XIa	9·0	10·12	11 244
XIIa	1·1	12·69	115 364
IIb	5·6	0·48	857
IVb	3·7	0·36	973
Vb	4·0	1·44	3 600
VIIIb	6·6	40·50	61 364

Michaelis constant, K_m, and maximal velocity of hydrolysis, v_{max}, were estimated according to Lineweaver and Burke[18] and checked according to Eisenthal and Cornish-Bowden[19]. The results are given in Table IV.

For interpretation we may apply the Michaelis–Menten equation.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[P_1]{k_2} ES \xrightarrow{k_3} E + P$$

Because only the amide group is splitted in our substrates we may introduce the simplifying conditions[20]: $k_1/k_{-1} = K_m$ and $k_2 = k_{cat}$. The Michaelis–Menten complex with chymotrypsin is bound by non-covalent hydrophobic interactions which are expressed in K_m values. This axiom is valid probably only for substrates in which the additional acyl or glycyl residue is localized in P_2 or in P_3 position. With decreasing hydrophobicity the K_m value increases in the series: Acetyl (*Ia*, 2·1), methoxyethoxyacetyl (*Ie*, 2·7), aminoacetyl (*IVa*, 4·2), hydroxyacetyl (*Id*, 5·0), 3-carboxy-2-propenoyl (*Ib*, 5·5) and 3-carboxypropanoyl (7·2, ref.[21]). The substrates with N-terminal glycine residue exhibit nearly equal K_m values: *IVa* 4·2, *VIIa* 4·3, *IVb* 3·7. The lowest K_m value (1·1) was observed with *XIIa* in which the histidine residue occupies position P_4. The k_{cat} values are similar for all substrates with varied acyl groups localized in positions P_2 and P_3: For *Ia*, *Ib*, *Id*, *IVa* 0·2 and 0·8 – 1·7, respectively, for *Va* 0·81, for *Vb* 1·44, for *VIIa* 1·68.

A remarkable change in k_{cat} values was found with substrates in which the anionic group is located in position P_4, *i.e.* with the 3-carboxypropanoyl tripeptides *VIIIa* and *VIIIb*, or in position P_5, *i.e.* with the 3-carboxypropanoyl tetrapeptide *XIa*. This is in agreement with our previous observation on 3-carboxypropanoylalanyl-alanyl-phenylalanine (or tyrosine) *p*-nitroanilides which are very good chymotrypsin substrates. Also *XIIa* with the histidine residue in position P_4 is a good substrate. We suppose that the imidazole ring is not protonated during the enzymic cleavage. It seems probable that in position P_4 only a bulky hydrophobic side chain can interfere with the chymotrypsin–substrate complex formation, *cf.*[22].

The optimal peptide chain length of the substrate in the phenylalanine series was reached with 3 amino acid residues for *VIIIa** $k_{cat}/K_m = 30000\ M^{-1}\ s^{-1}$, which is in accordance with other types of chymotrypsin substrates[23,24]. Therefore in the tyrosine series, the member containing the triglycyl unit was omitted from the synthetic scheme. The replacement of phenylalanine in position P_1 by tyrosine increases in all substrates twofold the k_{cat} value: *IVb*/*IVa* 1·7, *Vb*/*Va* 1·8, *VIIIb*/*VIIIa* 2·3. This regularity was also observed with acetylated peptide esters[26].

* During the preparation of this paper, the synthesis of *VIIIa* was published without analytical specification[25].

In contrast to the elastase the binding of chymotrypsin in the enzyme–substrate complex is not of electrostatic nature. This fact is evident from the present observation that also the shortest members in the 3-carboxypropanoyl series are good chymotrypsin substrates. Elastase is able to split only anionic substrates having the negagative group separated by three or more amino acid residues from the splitted bond[9].

EXPERIMENTAL

Melting points were determined on a Kofler block. Samples for elemental analyses were dried over phosphorus pentoxide at 105°C and 70 Pa. Compounds melting below 120°C were dried at room temperature. The $[\alpha]_D^{20}$ values were estimated on a Perkin–Elmer photoelectric polarimeter, concentrations 0·2—0·3 g/100 ml, unless stated otherwise. Solutions were taken down on a rotatory evaporator under diminished pressure. The standard working up of a reaction mixture implies evaporation under diminished pressure, dissolving of the residue in ethyl acetate, washing with 1M-HCl, water, 5% aqueous sodium hydrogen carbonate solution, and water, drying with sodium sulphate and evaporation. The analytical data of all compounds prepared in this paper are given in Tables *I*—*III*.

N-Acetylphenylalanine *p*-Nitroanilide (*Ia*)

To the solution of phenylalanine *p*-nitroanilide (2·85 g, 10 mmol) in pyridine (25 ml) acetanhydride (1·5 ml) was added at —5°C in the period of 30 min. The mixture was stirred for 30 min, evaporated and water was added to the residue. The solid was separated, yield 2·87 g (88%) of *Ia*, m.p. 243·246°C. The sample for analysis was crystallized from a 2-propanol–water mixture.

N-3-Carboxy-2-propenoylphenylalanine *p*-Nitroanilide (*Ib*)

The solution of phenylalanine *p*-nitroanilide (2·85 g, 10 mmol) and maleinanhydride (1 g, 10 mmol) in ethyl acetate (50 ml) was refluxed for 2 h and evaporated. The residue was crystallized from 2-propanol, yield 3·60 g (88%), m.p. 139—142°C after crystallization from 2-propanol.

N-Tert-butyloxyacetylphenylalanine *p*-Nitroanilide (*Ic*)

p-Nitrophenyl tert-butyloxyacetate (633 mg, 2·7 mmol) was added to the solution of phenylalanine *p*-nitroanilide (715 mg, 2·5 mmol) in dioxane. After 3 days standing the solution was evaporated and worked up (using 1% hydrochloric acid for washing). The residue was crystallized from ethyl acetate and light petroleum, yield 815 mg (82%) of *Ic*, m.p. 182—183°C. The sample for analysis was crystallized in the same manner.

N-Hydroxyacetylphenylalanine *p*-Nitroanilide (*Id*)

The solution of *Ic* (0·5 g, 1·25 mmol) in trifluoroacetic acid (5 ml) was left to stand for 3 h at room temperature, taken down to the half volume, diluted with diethyl ether (20 ml) and light petroleum (20 ml) was added. 280 mg (95%) of *Id* were collected, m.p. 180—184°C. The sample for analysis was crystallized from aqueous 2-propanol.

p-Nitrophenyl Methoxyethoxyacetate

Methoxyethoxyacetic acid (1·4 g, 10 mmol), *p*-nitrophenol (1·4 g, 10 mmol) and N,N′-dicyclohexylcarbodiimide (2·2 g, 10·7 mmol) were dissolved in dioxane (15 ml). N,N′-Dicyclohexylurea was filtered off and the solution was worked up. The residue was crystallized from a mixture of 2-propanol and light petroleum, 1·6 g (63%), m.p. 60—61°C which was not changed by repeated crystallization from the same solvent mixture. For $C_{11}H_{13}NO_6$ (255·3) calculated: 51·77% C, 5·13% H, 5·49% N; found: 51·68% C, 5·20% H, 5·34% N.

2,4,5-Trichlorophenyl Methoxyethoxyacetate

This ester was prepared in a similar manner as the *p*-nitrophenyl derivative, yield 67%, m.p. 57—58°C. The sample for analysis was crystallized from light petroleum, no change in the melting point value was observed. For $C_{11}H_{11}Cl_3O_4$ (313·6) calculated: 42·13% C, 3·54% H, 33·92% Cl; found: 42·48% C, 3·77% H, 33·31% Cl.

N-Methoxyethoxyacetylphenylalanine *p*-Nitroanilide (*Ie*)

Methoxyethoxyacetic acid (0·7 g, 5 mmol), phenylalanine *p*-nitroanilide (1·45 g, 5 mmol) and N,N′-dicyclohexylcarbodiimide (1·1 g, 5·3 mmol) were mixed in dioxane (10 ml). After 12 h standing N,N′-dicyclohexylurea was separated and the solution worked up, yield 1·25 g (63%) of *Ie*, m.p. 130—132°C after crystallization from ethyl acetate and light petroleum. The sample for analysis was crystallized in the same manner.

Benzyloxycarbonylglycyl-glycyl-phenylalanine *p*-Nitroanilide (*VIa*)

Benzyloxycarbonylglycyl-glycine (8·0 g, 30 mmol) and phenylalanine *p*-nitroanilide (8·6 g, 30 mmol) were dissolved in N,N-dimethylformamide (50 ml). To this solution N,N′-dicyclohexylcarbodiimide (6·6 g, 32 mmol) was added at 0°C. The mixture was stirred for 2 h at 0°C, left to stand for 10 h at room temperature and worked up. The crude product was crystallized from ethanol (125 ml) and water (50 ml), yield 10·6 g (67%) of *VIa*, m.p. 178—180°C. The sample for analysis was crystallized from ethyl acetate (saturated with water) and light petroleum. *IIIa*, *IXa* and *VIb* were prepared in a similar manner.

Glycyl-glycyl-phenylalanine *p*-Nitroanilide (*VIIa*)

The solution of *VIa* (8·0 g, 15 mmol) in acetic acid (20 ml) was treated with 35% HBr in acetic acid (20 ml), diethyl ether was added after 1 h standing, the separated solid was filtered off and dried over potassium hydroxide and phosphorus pentoxide. Sample for analysis was crystallized from methanol. The main portion of the crude product was dissolved in 50% aqueous ethanol (30 ml) and the base was set free using 1M sodium carbonate solution (30 ml). Crystallization from water yielded 2·53 g (42%) of *VIIa*.

Glycyl-glycyl-tyrosine *p*-Nitroanilide (*VIIb*)

The solution of *VIb* (2·2 g, 4 mmol) in acetic acid (4 ml) was treated with 35% HBr in acetic acid (4 ml), diethyl ether was added after 1 h standing, and the hydrobromide separated was dried over potassium hydroxide and phosphorus pentoxide, dissolved in water (10 ml) and the base set free by addition of 10% aqueous ammonia (5 ml). The product was crystallized from

water (80 ml); yield 960 mg (58%), m.p. 197—201°C. Sample for analysis was recrystallized from water. *IVa*, *IVb* and *Xa* were prepared in the same way.

3-Carboxypropanoylglycyl-glycyl-phenylalanine *p*-Nitroanilide (*VIIIa*)

Succinanhydride (500 mg, 5 mmol) was added to the solution of *VIIa* (600 mg, 1·5 mmol) in N,N-dimethylformamide (5 ml). The mixture was heated for 2 h at 80°C, evaporated and the residue was triturated with water (5 ml). The solid was crystallized from a mixture of water (20 ml) and methanol (15 ml); yield 620 mg (83%), m.p. 211—212°C. *IIb*, *Va*, *Vb*, *VIIIb* and *XIa* were prepared in a similar manner.

N^{α}-Acetylhistidyl-glycyl-glycyl-phenylalanine *p*-Nitroanilide (*XIIa*)

The solution of N^{α}-acetylhistidine hydrazide (490 mg, 2·3 mmol) in N,N-dimethylformamide (40 ml) and concentrated hydrochloric acid (0·93 ml) was treated at —12°C with a sodium nitrite (160 mg) solution in water (0·65 ml). The mixture was stirred for 10 min at —12°C, then the pH was adjusted to 6·9 with N-ethylpiperidine and the mixture was added to the solution of *VIIa* (800 mg, 2 mmol) in N,N-dimethylformamide (15 ml). After 12 h standing at +3°C the solution was evaporated, the residue ground with dichloromethane (80 ml), the solid material filtered off, dissolved in methanol (15 ml) and purified on a silica gel column using dichloromethane–methanol (3 : 1) as eluent. The combined homogeneous fractions (positive to Pauly reagent) were evaporated and the residue was crystallized from a mixture of N,N-dimethylformamide, 2-propanol and diethyl ether. Yield 280 mg (23%).

N^{α}-Acetylhistidine Hydrazide

The solution of histidine methyl ester dihydrochloride (24·2 g, 0·1 mol) in water (150 ml) was adjusted by sodium hydrogen carbonate to pH 6·5, cooled down to 0°C and acetanhydride was added in the period of 15 min. The pH value was maintained at 6·5 by addition of sodium hydrogen carbonate. After 30 min of stirring at room temperature the solution was acidified (4M-HCl) to pH 2 and evaporated. The residue was dissolved in methanol (150 ml), sodium chloride which separated was filtered off and the solution taken to dryness. The residue (crude N^{α}-acetylhistidine methyl ester) was dissolved in methanol (100 ml), 80% hydrazine hydrate (50 ml) was added and after 12 h standing at room temperature the crude hydrazide was filtered off. Crystallization from ethanol–diethyl ether mixture yielded 7·35 g (35%) of a product melting at 206 to 210°C. The sample for analysis was crystallized from aqueous methanol, m.p. 224—226°C. For $C_8H_{13}N_5O_2$ (211·2) calculated: 45·06% C, 6·20% H, 33·16% N; found: 45·50% C, 6·38% H, 33·04% N.

Enzymic Activity Measurements

The K_m and v values were calculated from initial velocities of hydrolysis using 5 or 6 different concentrations of substrates in the incubation medium containing 1·8 ml of 50 mM Tris.HCl buffer (pH 7·8), 100 μml substrate solution in dimethylformamide and 100 μml of the chymotrypsin (SPOFA, Czechoslovakia) solution in 4mM-HCl and 0·5 mM-$CaCl_2$. The reactions were performed in thermostated photometric cells at 25°C. The amount of liberated *p*-nitroaniline was estimated by continual recording of absorbance at 405 nm using SPEKOL apparatus and recorder K 200 (Zeiss, Jena). Concentrations of substrates in incubation media were as follows: 1 mM—0·1 mM for *Ia*, *Ib*, *Id*; 0·5 mM—0·1 mM for *IVa*, *Va*, *VIIa*, *VIIIa*, *XIa*, *IIb*, *IVb*, *Vb*, *VIIIb*;

0·25 mM—0·05 mM for *XIIa*; 0·125 mM—0·025 mM for *Ie*. Different concentrations were used because of the differences in solubility of single substrates. Actual concentrations of chymotrypsin in incubation media were as follows: 5·926 μM for *Ia*, *Ib*, *Ie*, *Id*; 3·951 μM for *IVa*, *Va*, *IIb*, *IVb*, *Vb*; 1·975 μM for *VIIa*; 0·197 μM for *VIIIa*, *XIIa*, *VIIIb*.

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