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The Action of Trypsin on Synthetic Substrates. ε-N-Methyl-L-lysines and Lysine Homologues

Thesis presented by

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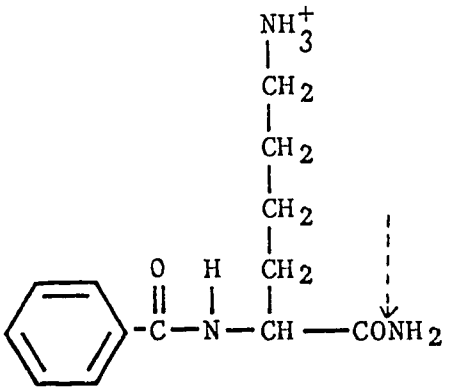
Introduction

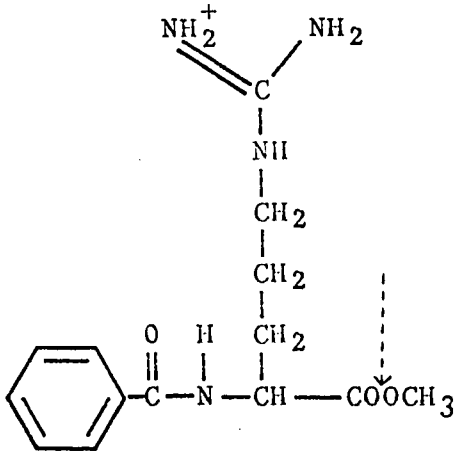
Trypsin is an old name which referred to the general enzymatic activity of pancreatic juice. When several proteolytic enzymes were characterized in this juice, the meaning of the word trypsin became restricted to a single enzyme belonging to the endopeptidase group. The enzyme is synthesized by pancreas in the form of an inactive precursor called trypsinogen. The activation of this zymogen, which involves the cleavage of a hexapeptide from one end of the protein chain, may be done by trypsin itself, or by enterokinase. Northrup et al. (1) were able to show that the kinetic curve of the enzyme activation was S-shaped, indicating that the reaction was auto-catalytic.

Trypsin (E.C. 3.4.4.4.) was among the first enzymes to be obtained in a crystalline state. The isolation and crystallization of bovine trypsin was described by Northrup (2) and Northrup and Kunitz (3). Since that time the specificity of trypsin has been under continuous investigation. The first meaningful specificity studies were undertaken by Bergmann, Fruton and Pollok in 1939 (4). This early work established the high specificity of the enzyme for the amide derivatives of L-lysine and L-arginine. These original conclusions on the specificity of trypsin were supported by subsequent studies on ester as well as amide substrates by Schwert et al. (5). The natural substrates for this enzyme are proteins, but the discovery

that small molecules could be used instead led to the detailed study of enzyme action. The location of the bond hydrolyzed by trypsin is shown below for two typical substrates.



α-N-Benzoyl-L-lysinamide



α-N-Benzoyl-L-arginine methyl ester

The pH optimum of the enzyme lies between 6 and 11 depending upon the nature of the substrate used. In this region the enzyme is quite unstable because of self-degradation. This instability can be prevented by the addition of a suitable cation e.g. calcium, magnesium, or manganese (6).

Trypsin belongs to a large group of enzymes that have one especially reactive serine hydroxyl group among the 20 to 30 serine residues of the protein molecule. The enzymes of this group all catalyze acyl or phosphoryl transfer reactions, hydrolysis being a special case of transfer to water as acceptor. The reactive serine was

discovered when diisopropylphosphofluoridate (DFP) was reacted with trypsin. One molecule of the DFP reagent was found to combine with one molecule of the enzyme. Furthermore, when DFP-treated trypsin is hydrolyzed with acid (7) or with enzymes (8,9) the phosphoryl group is found on a serine residue which belongs to the Gly-Asp-Ser-Gly sequence common to the active site of many esterases. This observation, and the fact that trypsin is reversibly inactivated by formylation of these serine and threonine residues (10), suggest that one serine residue of the chain is involved in the active center.

The pH dependence curve of trypsin is consistent with the view that a group with a pK of 6.25 is involved in the catalysis (11). Thus, in the present state of our knowledge, the catalytic power of trypsin appears to be determined by the concerted action of a serine and a histidine residue.

Trypsin appears to have a molecular weight of 23,800 as estimated by sedimentation in the ultracentrifuge. This value is in good agreement with that found by measuring the phosphorus content of DFP-treated trypsin.

Since one of the objects of our work was to measure the approximate Michaelis-Menten constants for several new substrates, the following kinetic outline is given:

The original mechanism, as proposed by Michaelis and Menten (12), allowed for the formation of an enzyme-substrate

complex. In this scheme the enzyme E attacks the substrate S to give the enzyme-substrate complex ES. This complex may then break down, releasing the free enzyme and the product P.

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

The rate of the reaction is given by:

$$V = \frac{k_2 [E_o][S]}{\frac{k_2 + k_{-1}}{k_1} + [S]}$$

where $[E_o]$ is the initial enzyme concentration.
The combination of rate constants in the denominator

$$\frac{k_2 + k_{-1}}{k_1} = Km$$

is the Michaelis-Menten constant. This treatment makes the unjustified assumption that Km is an equilibrium constant.

Briggs and Haldane (13) proposed that the Michaelis-Menten treatment was too limited, and substituted the assumption that, after a brief initial period during which the concentration of intermediates builds up, a steady state is reached. Some of the important assumptions are 1) $[S] \gg [E]$, 2) the rates of utilization of substrate and appearance of products are equal and constant over the period of the rate measurement, and 3) the concentrations of enzyme intermediates are constant over the period of

the rate measurement, or at least their rates of change are small compared with the rate of the overall reaction (14). The kinetic treatment of the simple one-intermediate scheme by the steady state method is shown below:

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

The steady state demands that

$$k_1 [E][S] - k_{-1} [ES] - k_2 [ES] = 0$$

Since there is conservation of the total number of enzyme sites,

$$[E_o] = [E] + [ES]$$

Expressing in terms of ES we obtain

$$[E_o] = ES \quad \frac{k_{-1} + k_2}{k_1 [S]} + 1 \qquad (1)$$

The initial velocity is given by

$$V = k_2 [ES] \qquad (2)$$

Substituting (1) in (2) we obtain

$$V = \frac{k_2 [E_o]}{\frac{k_{-1} + k_2}{k_1 [S]} + 1} = \frac{k_2 [E_o][S]}{Km + [S]} \qquad (3)$$

Equation (3) is identical with the rate expression obtained from the Michaelis-Menten equilibrium constant. This equation also suggests a number of methods for determining k_2 and Km by graphical methods. For example a plot of 1/V vs 1/S (Lineweaver-Burk) would give a straight line of slope Km/V_{max} and an intercept of $1/V_{max}$.

A more useful procedure is to plot S/V vs S (modified Eadie, Woolf) which gives a straight line of slope $1/V_{max}$ with an intercept of Km/V_{max}. The Lineweaver-Burk plot is not as useful because the experimental points tend to cluster at high substrate concentrations.

In the case of trypsin there is evidence for the existence of two intermediates in the reaction scheme. Under these circumstances, Km becomes more complex than in the case shown above. For example, if the reaction proceeds via two intermediates,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[P_1]{k_2} ES' \xrightarrow{k_3} E + P_2$$

the following derivation of Km results:
If we solve this equation by a steady-state treatment the initial velocity becomes

$$V = \frac{\dfrac{k_2 k_3}{k_2 + k_3}\,[E_o][S]}{\dfrac{k_{-1} + k_2}{k_1} \cdot \dfrac{k_3}{k_2 + k_3} + [S]}$$

The above equation has the same form as the Michaelis-Menten equation, but now there are more rate constants in each term,

$$Km = \frac{k_{-1} + k_2}{k_1} \cdot \frac{k_3}{k_2 + k_3}$$

For this reason, the Km value obtained from such a scheme is called Km apparent or experimental (Km app). The expression

$$\frac{k_2 k_3}{k_2 + k_3}$$

is known as k_{cat}. In the case of the hydrolysis of an ester by trypsin it is known that k_3 (deacylation) is rate limiting. Under these conditions k_{cat} becomes equivalent to k_3 at high substrate concentrations. The inclusion of pH effects can increase the complexity of Km considerably.

Although the three-dimensional structure of trypsin has not yet been determined, the structure of chymotrypsin has been available for some time (15). The amino acid sequences of trypsinogen and chymotrypsinogen (16) are remarkably similar over long stretches. One of the more notable differences, however, is the change of serine 189 in chymotrypsin for an aspartic acid residue at the corresponding position, 177, in trypsin. If one assumes that trypsin has the same general conformation as does chymotrypsin, then this aspartic acid residue would be about 7 angstroms from the active center of the enzyme. It has already been suggested that this is the anionic site to which the positively-charged side-chain of a substrate is bound (17).

A great number of small model compounds have been used to study the specificity of trypsin. The selection of our substrates was based upon the results, summarized in Table I, which were obtained using these compounds. The abbreviations used throughout the table are those of the IUPAC-IUB commission on Biochemical Nomenclature (18) where possible. In some cases no abbreviation has been selected by the commission and the following were used:

a) Amino Acids

Homoarginine	Homoarg
Homolysine	Homolys
Norarginine	Norarg
Oxalysine	Oxalys
Thialysine	Thialys

b) N-protecting Groups

β-Carboxyproprionyl	BCP
Formyl	For
Nicotinyl	Nic

c) Carboxyl-protecting Groups

Butyl ester	OBu
Cyclohexyl ester	OCycloh
Hexyl ester	OHex
Isoamyl ester	OIsoam
Isopropyl ester	OIsopr

β-Napthyl ester	ONapth
Pentyl ester	OPtyl
Propyl ester	OPr
3-Pyridyl ester	OPyr
Amide	NH_2
Anilide	An
Dichloroanilide	Dichloroan
Hydrazide	$NHNH_2$
Methoxyanilide	Methan
α-Napthylamide	αNa
β-Napthylamide	βNa
p-Nitroanilide	pNA

The compounds were grouped into four main categories 1) derivatives of lysine, 2) derivatives of arginine, 3) pseudo substrates and 4) non-specific substrates. Group 3 are those compounds which bear a structural relationship to either lysine or arginine. These groups were divided into sub-groups depending upon the number of amino acid residues which the compounds contained. Within these sub-groups, the compounds are listed alphabetically by unabbreviated name, and occasionally in order of increasing complexity. The table does not list protein substrates.

A number in the "remarks" section refers to the literature Km at the given pH unless otherwise specified.

The symbols in the rate column give semi-quantitatively the susceptibility of the compound to trypsin. The following symbols are used:

0	not hydrolyzed
±	hydrolyzed very slowly
+	hydrolyzed slowly
++	hydrolyzed at an appreciable rate
+++	hydrolyzed very rapidly

Table I

The Action of Trypsin on Synthetic Compounds

#	Compound	Rate	Remarks	Ref.
	LYSINES			
1	Lys-OBzl	++		19
2	Lys-OBu	++		19
3	Lys-OEt	++		19-23
4	Lys-OMe	++	9.3×10^{-3} pH 8.0	19,24,25
5	Lys-OPtyl	++		19
6	Lys(Me)-OEt	+		26
7	Lys(Z)-OMe	0		5
8	Lys-NH_2	+		27,28
9	Lys-pNA	+	3.6×10^{-4} pH 8.65	29
10	Ac-Lys-OEt	+++		24
11	Ac-Lys-OMe	+++	2.5×10^{-4} pH 7.0	30
12	Ac-D-Lys-OMe	+	2.0×10^{-3} pH 7.0	30
13	Ac-Lys(Me,Me)-OEt	0		24
14	Bz-Lys-OCycloh	+++	2.95×10^{-5} pH 8.0	31
15	Bz-Lys-OEt	+++		32
16	Bz-Lys-OHex	+++		33
17	Bz-Lys-OIsoam	+++		33
18	Bz-Lys-OIsopr	+++		33
19	Bz-Lys-OMe	+++	5.5×10^{-5} pH 7.0	30,33,34
20	Bz-Lys-NH_2	++	4.6×10^{-3} pH 8.0	27,35-37

#	Compound	Rate	Remarks	Ref.
21	Bz-Lys(Me)-NH_2	+		26
22	Bz-Lys-$NHNH_2$	++		38
23	Z-Lys-OBzl	+++	1 x 10^{-4} pH 5.80	39
24	Z-Lys-OMe	+++	2.98 x 10^{-4} pH 5.80	39
25	Z-Lys-ONp	+++	1.0 x 10^{-5} pH 5.80	39-42
26	MeLys-OEt	0		21
27	Tos-Lys-OEt	+++	3.63 x 10^{-5} pH 8.0	31,43
28	Tos-Lys-OMe	+++	4.18 x 10^{-5} pH 8.0	33
29	Tos-Lys-ONapht	+++		31
30	Tos-Lys-OPr	+++	2.2 x 10^{-5} pH 8.0	33
31	Tos-Lys-OPyr	+++		31
32	Lys-Lys	+		27,28
33	Abu-Lys-OMe	++		44
34	Gly-Lys-OMe	++		44
35	Leu-Lys-OMe	++		44
36	Lys-Lys-OEt	++	5 x 10^{-4} pH 7.6	45,46
37	Nle-Lys-OMe	++		44
38	Phe-Lys-OMe	++		44
39	Val-Lys-OMe	++		44
40	Abu-Lys-NH_2	++		44
41	Ala-Lys-NH_2	++		44
42	βAla-Lys-NH_2	++		44
43	Gly-Lys-NH_2	++		27,44
44	Leu-Lys-NH_2	++		44
45	Lys-Gly-NH_2	+		27
46	Lys-Lys-NH_2	+		27

#	Compound	Rate	Remarks	Ref.
47	Phe-Lys-NH_2	++		44
48	Bz-Lys-Lys	+		27
49	Bz-Gly-Lys-OMe	++	1.7×10^{-4} pH 7.0	30
50	Bz-Gly-Lys-NH_2	++		47
51	Bz-Gly-Lys(Z)-NH_2	0		36
52	Pht-Gly-Lys-OMe	++	1.0×10^{-4} pH 6.5	48
53	Ala-Lys-Ala	++		49
54	Lys-Lys-Lys	++	no free lysine formed	27
55	Lys-Tyr-Leu	++		28
56	Lys-Tyr-Lys	++		28
57	Tyr-Lys-Glu	0		47
58	Lys(Tos)-Val-Tyr-OEt	±		50
59	Pro-Val-Lys(Tos)-OMe	±		50
60	Val-Lys(Tos)-Val-OMe	±		50
61	Gly-Gly-Lys-NH_2	++		51-52
62	Lys-Lys-Tyr-NH_2	++		28
63	Bz-Lys-Lys-Lys	++		27
64	Z-Lys(Z)-Arg(NO_2)-Pro-OMe	0		50
65	Z-Pro-Ser-Lys(For)-OMe	±		50
66	Gly-Gly-Lys-Gly	++	1.96×10^{-2} pH 8.5	52
67	Lys-Lys-Lys-Lys	++		27
68	Lys-Lys-Glu-Lys	+		59
69	Pyroglu-Pro-Ser-Lys(Boc)-OMe	0		50
70	Val-Lys(Tos)-Val-Tyr-OEt	±		50

#	Compound	Rate	Remarks	Ref.
71	Gly-Gly-Gly-Lys-NH_2	++		50
72	Gly-Gly-Lys-Gly-Gly	++	8.9×10^{-3} pH 8.5	52
73	Tyr-Tyr-Lys-Glu-Tyr	++		47
74	Gly-Gly-Gly-Gly-Lys-NH_2	++		51
75	Gly-Gly-Lys-Gly-Gly-Gly	++	3.5×10^{-3} pH 8.5	52
76	Gly-Gly-Lys-Gly-Gly-Gly-Gly	++	1.7×10^{-3} pH 8.5	52
77	Z-Lys(Z)-Arg(NO_2)-Pro-Phe-Ser-Pro-Phe-Arg(NO_2)-OMe	0		50
78	R-Lys(Isopr)-R'	+		54
79	$(D\ Lys)_n$	0		55
80	$(Lys)_n$	++		56-61
81	$(Lys(purinyl))_n$	+	partially alkylated	62
	ARGININES			
82	Arg-OMe	++		63
83	Bz-Arg-OBzl	+++		64
84	Bz-Arg-OCycloh	+++		64
85	Bz-Arg-OEt	+++	1.0×10^{-5} pH 8.0	47,64-88
86	Bz-Arg-OIsopr	+++		64

#	Compound	Rate	Remarks	Ref.
87	Bz-Arg-OMe	+++	9.6 x 10^{-6} pH 7.0	30,47,64-65,89-91
88	Bz(oNO_2)-Arg-OMe	+++		91
89	Bz(mNO_2)-Arg-OMe	+++		91
90	Bz(pNO_2)-Arg-OMe	+++		91
91	Bz(3,5-diNO_2)-Arg-OMe	+++		91
92	Bz-DL-Arg-An	++		92
93	Bz-Arg-NH_2	++	2.1 x 10^{-3} pH 8.65	29,35-37,47,64,93-107
94	Bz-DL-Arg-NH_2	++		94
95	Bz-DL-Arg-Dichloroan	++		92
96	Bz-DL-Arg-Methan	++		92
97	Bz-DL-Arg-αNa	++		92
98	Bz-Arg-βNa	++		92,107-110
99	Bz-DL-Arg-βNa	++		110-111
100	Bz-DL-Arg-pNA	++		29,112-115
101	Bz-D-Arg-pNA	0	Ki 8.0 x 10^{-4}	29
102	BCP-Arg-βNa	++		92
103	Z-Arg-OMe	+++		108
104	Z-Arg-βNa	++		92,108
105	Z-Arg(NO_2)-NH_2	0		104
106	Tos-Arg-OCycloh	+++	8.2 x 10^{-6} pH 8.4	116
107	Tos-Arg-OEt	+++		116
108	Tos-Arg-OMe	+++	6.4 x 10^{-6} pH 8.4	5,30,33,65,66,76,85, 107,116-127
109	Tos-D-Arg-OMe	++	2.7 x 10^{-4} pH 8.0	120
110	Tos-Arg-OPr	+++	6.6 x 10^{-6} pH 8.4	65,116
111	Tos(2,4-diNO_2)-Arg-OMe	+++		128

#	Compound	Rate	Remarks	Ref.
112	Tos(Et)-Arg-OMe	+++		128
113	Tos(Isopr)-Arg-OMe	+++		128
114	Tos(MeO)-Arg-OMe	+++		128
115	Tos(NO_2)-Arg-OMe	+++		128
116	Tos-Arg-NH_2	++	7.0×10^{-3} pH 8.0	5,76,99,103
117	Arg-Gly	++		104
118	Arg-Glu	++		104
119	Arg-Leu	++		104
120	Arg-Phe	++		104
121	Arg-Arg-βNa	++		92
122	Gly-Arg-βNa	++		92
123	Bz-Gly-Arg-NH_2	++		47
124	Bz-Gly-Arg(Z)-NH_2	0		47
125	BCP-Arg-Arg-βNa	++		92
126	Z-Arg-Arg-An	++		92
127	Z-Arg-Arg-NH_2	++		92
128	Z-Arg-Arg-βNa	++		92
129	Z-Arg(NO_2)-Leu	0		104
130	Z-Arg(NO_2)-Phe	0		104
131	Arg-Arg-Arg-βNa	++		92
132	Gly-Gly-Arg-βNa	++		92
133	BCP-Gly-Gly-Arg-βNa	++		92
134	Z-Arg(NO_2)-Arg(NO_2)-Ala-OBzl	±		50
135	Z-Arg-Arg-Arg-An	++		92

#	Compound	Rate	Remarks	Ref.
136	Z-Arg-Arg-Arg-NH	++		92
137	Z-Arg-Arg-Arg-βNa	++		92
138	Z-Arg(NO_2)-Leu-Glu(OEt)-OEt	±		50
139	Z-Gly-Arg-Arg-βNa	++		92
140	Z-Gly-Gly-Arg-βNa	++		92,129
141	Z-Lys(Z)-Arg(NO_2)-Pro-OMe	0		50
142	Arg-Pro-Pro-Gly-OMe	0		50
143	Z-Arg(NO_2)-Pro-Pro-Gly-OMe	0		50
144	Z-Gly-Arg-Arg-Arg-βNa	++		92
145	Boc-Leu-Asp(NH_2)-Ser-Arg(NO_2)-Arg(NO_2)-Ala-OBzl	±		50
146	Z-Asp(NH_2)-Arg(NO_2)-Tyr-Val-Val-His-Pro-Phe-OBu^t	±		50
	PSEUDO-SUBSTRATES			
147	βAla-Gly-OMe	++		24
148	Bz-Ser(Gly)-OEt	++		24
149	Z-Glu($NHNH_2$)-OEt	+	7.1×10^{-2} pH 7.5	32
150	$(Glu(\gamma NHNH_2)_n$	0		32
151	Z-Glu($NHNH_2$)-NH_2	±		4,32
152	Bz-Dbu-NH_2	±		34,94

#	Compound	Rate	Remarks		Ref.
153	Bz-Orn-NH_2	±			34
154	Bz-Orn(pyrimidinyl)-OEt	0			130
155	Tos-Orn-OEt	+	1.9×10^{-2}	pH 6.75	30
156	Tos-Orn-OMe	+	1.53×10^{-2}	pH 7.0	116
157	Tos-DL-Orn-OMe	+			116
158	$(Orn)_n$	0			47
159	Tos-Norarg-OMe	++	6.94×10^{-4}	pH 8.0	131
160	Tos-Norarg-OPr	++	5.31×10^{-4}	pH 8.0	131
161	Ac-Thialys-OEt	++	1.0×10^{-3}	pH 8.0	24
162	Bz-Thialys-OCycloh	+++	2.95×10^{-5}	pH 8.0	31
163	Bz-Thialys-OMe	+++	9.41×10^{-5}	pH 8.0	31
164	Bz-Thialys-NH_2	++	4.3×10^{-3}	pH 8.0	35
165	Tos-Thialys-OEt	+++	3.69×10^{-4}	pH 8.0	31
166	Tos-Thialys-OMe	+++	2.16×10^{-4}	pH 8.0	31
167	Tos-Thialys-OPr	+++	2.65×10^{-4}	pH 8.0	31
168	Pht-Gly-Thialys-OMe	++	4.0×10^{-4}	pH 6.5	48
169	Ac-Cysteine(Carboamidomethyl)-OEt	+	4.0×10^{-2}	pH 8.0	24
170	Pht-Ala-Oxalys-OMe	+	2.0×10^{-2}	pH 6.5	48
171	Pht-Gly-DL-Oxalys-OMe	+	1.0×10^{-2}	pH 6.5	48

#	Compound	Rate	Remarks		Ref.
172	Methyl-α-hydroxy-γ-guanidino-valerate	+			132
173	Bz-Citrulline-OMe	+	4.1×10^{-2}	pH 7.0	30,133
174	Tos-Citrulline-OMe	+	9.1×10^{-2}	pH 7.0	30
175	Bz-DL-Homoarg-OEt	+			131
176	Tos-Homoarg-OCycloh	++	2.38×10^{-4}	pH 8.4	116
177	Tos-Homoarg-OEt	++	2.89×10^{-4}	pH 8.4	116
178	Tos-Homoarg-OMe	++	3.32×10^{-4}	pH 8.4	116
179	Tos-Homoarg-OPr	++	2.57×10^{-4}	pH 8.4	116
NON SPECIFIC					
180	Gly-OEt	0			5
181	His-OMe	+	3.0×10^{-2}	pH 8.0	24
182	Leu-OEt	0			63
183	Leu-ONapht	0			47
184	Phe-OEt	±			5
185	Tyr-OEt	±			5,134
186	Tyr-NH_2	0			47
187	Ac-Gly-OMe	±	1.4	pH 7.0	30,135
188	Ac-Gly-OEt	±	0.79	pH 6.6	136,137
189	Ac-Phe-OMe	±			76
190	Ac-DL-Phe-ONapht	±			138
191	Ac-Tyr-OEt	+			76,139
192	Ac-Tyr-βNa	±			140
193	Ac(Cl)-Leu	0			47
194	Ac(Cl)-Tyr	0			47

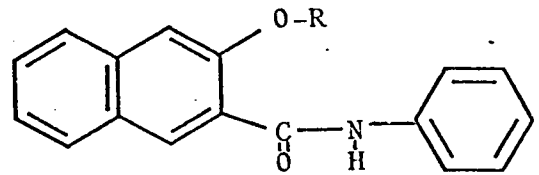
#	Compound	Rate	Remarks	Ref.
195	Bz-Gly-OMe	0		141
196	Bz-Gly-NH_2	0		47
197	Bz-Glu-NH_2	0		47
198	Bz-His-NH_2	0		47
199	Bz-Norleu-OEt	0		47
200	Bz-Norval-OEt	0		47
201	Bz-DL-Phe-ONapht	±		138,142
202	Bz-Tyr-NH_2	0		47
203	Z-Glu-NH_2	0		47
204	Z-Tyr-ONp	+		143
205	N-(3-Carboxy-propriony1)-Phe-pNA	+		113
206	N-(Dimethylamino-acetyl)-Phe-pNA	+		113
207	N-(3-Methoxy-carboxy-propriony1)-Phe-pNA	+		113
208	Nic-DL-Phe-p-anisidine	0		138
209	Nic-Phe-βNa	0		138
210	Nic-DL-Phe-βNa	0		138
211	Nic-DL-Phe-βNa(N-methiodide)	0		138
212	Pht-DL-Phe-ONapth	0		138
213	Trit-Ser-OMe	0		50
214	Ala-Phe-OMe	±		50
215	Gly-DL-Ala	0		47
216	Gly-Asp	0		47
217	Gly-Gly	0		47
218	Gly-Tyr	0		47

#	Compound	Rate	Remarks	Ref.
219	Leu-Gly	0		47
220	Leu-Leu	0		47
221	Leu-Tyr	0		47
222	Thr-Phe-OMe	+		47
223	Val-Leu-OMe	±		50
224	Ac-Phe-Ser(Ac-Phe)	±		50
225	Bz-Norleu-alloThr(Bz)	±		144
226	Bz-Norleu-DL-Thr(Bz)	±		144
227	Bz-Norleu-Ser(Bz)-$NHCH_3$	±		144
228	Bz-Norleu-alloThr(Bz)-$NHCH_3$	±		144
229	Bz-Norleu-DL-Thr(Bz)-$NHCH_3$	±		144
230	Bz-Phe-Ser(Bz)	±		144
231	Bz-Phe-Ser(Bz-Phe)	±	5% hydrolysis	145
232	Bz-Phe-alloThr(Bz)	±	in 2 hours	145
233	Bz-Phe-DL-Thr(Bz)	±		144
234	Bz-Phe-alloThr(Bz)-$NHCH_3$	±		144
235	Bz-Phe-DL-Thr(Bz)-$NHCH_3$	±		144
236	Bz-Ser(Bz-Ala)-OEt	±		145
237	Bz-Ser(Bz-Phe)-OEt	±		145
238	Bz-Tyr-Gly-NH_2	0		47
239	Bz-Val-Ser(Bz)	±		144
240	Bz-Val-DL-Thr(Bz)	±		144
241	Bz-Val-Ser(Bz)-$NHCH_3$	±		144
242	Bz-Val-alloThr(Bz)-$NHCH_3$	±		144
243	Bz-Val-DL-Thr(Bz)-$NHCH_3$	±		144

#	Compound	Rate	Remarks	Ref.
244	Z-Gly-DL-Phe-OEt	0		5
245	Z-Gly-Tyr-OEt	0		5
246	Z-Try(Ac)-Gly-OEt	±		5
247	Z-Tyr-Gly-NH_2	0		47
248	Z-Val-Gly-OMe	0		50
249	Z-Val-His-OMe	±		50
250	Z-Val-Leu-OBzl	±		50
251	Pyroglu-Glu(OEt)-OEt	±		50
252	Ala-Gly-Gly	0		47
253	Gly-Gly-Gly	0		47
254	Leu-Gly-Gly	0		47
255	Ala-Ala-Ala-OEt	±		50
256	Ala-Ala-Phe-OMe	±		50
257	Ala-Ala-D-Phe-OMe	0		50
258	Asp(NH_2)-Ala-Phe-OMe	±		50
259	Asp(NH_2)-Phe-Val-OMe	±		50
260	Leu-Val-Asp(OEt)-OEt	±		50
261	Leu-Val-D-Glu(OEt)-OEt	0		50
262	Phe-Ala-Phe-OMe	±		50
263	Pro-Phe-Gly-OMe	±		50
264	Boc-Leu-Gly-Leu-OBu^t	±		50
265	Z-Asp(NH_2)-Phe-Val-OMe	0		50
266	Z-Gly-Tyr-Gly-NH_2	0		47
267	Z-Leu-Val-Asp(OEt)-OEt	±		50

#	Compound	Rate	Remarks	Ref.
268	Ala-Ala-Ala-Ala	0		146
269	Gly-Gly-Gly-Gly	0		47
270	Ala-Ala-Phe-Val-OMe	±		50
271	Ala-Phe-Ala-Phe-OMe	+		50
272	Glu(NH_2)-Gly-Thr-Phe-OMe	±		47
273	Leu-Met-Asp(NH_2)-Thr-OMe	±		47
274	Val-His-Pro-Phe-OBut	±		47
275	Z-Ser-Tyr-Ser-Met-OMe	±		47
276	DL-Ala-DL-Ala-DL-Ala-DL-Ala-DL-Ala	0		146
277	Gly-Gly-Gly-Gly-Gly	0		146
278	Ala-Ser-Glu(NH_2)-Tyr-Ser-OMe	±		50
279	Thr-Ser-Asp(NH_2)-Tyr-Ser-OMe	±		50
280	Phe-Ser-Pro-Phe-Ala-OMe	±		50
281	Gly-Gly-Gly-Gly-Gly-Gly	0		47
282	N-(Bzlnicamidomethyl)-N'-βnapthylurea	0		138
283	Butyl acetate	0		5
284	N-trans-cinnamoyl imidazole	+		147
285	Ethyl p-amidinobenzoate	+		148
286	Ethyl butyrate	0		5
287	β-Napthyl acetate	±		149
288	p-Nitrophenyl acetate	+		150
289	p-Nitrophenyl p-amidobenzoate	+		148
290	$H_2N-(CH_2)_3-COOCH_3$	0		151
291	$H_2N-(CH_2)_4-COOCH_3$	±		151

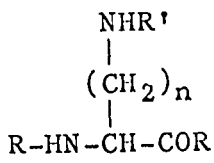
#	Compound	Rate	Remarks	Ref.
292	εAcp-OMe	+		94,151
293	$H_2N-(CH_2)_5-CO-CH_2-COOC_2H_5$	±		151
294	Ac-heptylene-OMe	+	4.9×10^{-2} pH 7.0	30
295	Bz-heptylene-OMe	+	1.0×10^{-4} pH 7.0	30
296	Methyl α-acetamidoadipate	+	4.5×10^{-3} pH 8.0	24
297	Poly-(ε-aminocaproyl)-amino acids	±		152



298 R =

	$-CO-C_6H_5$	±	153
299	$-CO-CH_2-C_6H_5$	±	153
300	$-CO-CH_2-CH_2C_6H_5$	±	153
301	$-CO-CH_2-CH_2-CH_2C_6H_5$	±	153
302	$-CO-CH_2CH_3$	±	153
303	$-CO-CH_2CH_2Cl$	±	153

The structural requirements for trypsin substrates may be summarized with the aid of the following model:



In the case of lysine n = 4 and R' is H, while in the case of arginine n = 3 and R' is $-C(NH_2)=NH$.

1) Substitution on the α-amino group (R) by an acyl moiety is not absolutely required, since compounds without any subtituent in this position are readily hydrolyzed. For example, L-lysine ethyl ester and similar compounds are good substrates (# 1-5, 82), even though they are hydrolyzed more slowly than the acylated derivatives (# 14-19, 27-31, 83-90, 106-115). The presence of an alkyl group on the α-nitrogen seems to prevent hydrolysis entirely, since α-N-methyl-L-lysine ethyl ester (# 26) is resistant to trypsin. The removal of the amino group altogether considerably reduces the susceptibility of the compound to hydrolysis (# 292). The nature of an amino acid residue linked to the α-nitrogen, e.g. as is found in a peptide, influences the rate of hydrolysis very little (# 33-47).

2) Substitution on the ε-amino group (R') of lysine or on the amino group of the guanidinium moiety of arginine

by an acyl residue prevents hydrolysis. Similarly, derivatives containing nitroarginine are resistant to the enzyme. The only exception to this is found in the work of Kloss and Schröder (50) who showed that under certain circumstances formyl and tosyl lysines as well as nitroarginines are slowly hydrolyzed (# 58-60, 65, 134, 138, 145, 146). It has been shown that an alkyl substituent on the ε-amino group (R') does not prevent hydrolysis, since ε-N-methyl-L-lysine ethyl ester (# 6) is hydrolyzed by trypsin. The benzoyl amide of ε-N-methyl-L-lysine (# 21) is also hydrolyzed. At the outset of our work no data were available on other alkylated lysines or on any alkylated arginines.

3) The residue near the susceptible bond (R") may be a peptide, an amide or an ester. Esters are hydrolyzed more rapidly than either amides or peptides. The nature of the alcohol forming the ester bond does not affect the kinetic parameters Vmax or Km greatly (# 23-25, 27, 28, 30). The type of amide present also does not appear to affect the rate of hydrolysis (# 93-99). The nature of the amino acid residue in a peptide does affect the rate of hydrolysis considerably. There is evidence to suggest that a negative charge in the vicinity of the susceptible bond tends to retard tryptic hydrolysis, since L-tyrosyl-L-lysyl-L-glutamyl-L-tyrosine (# 68) is hydrolyzed slowly,

while L-tyrosyl-L-lysyl-L-glutamic acid (# 57) is not hydrolyzed at all.

4) Several other variations are possible within the model itself. The replacement of a methylene group in the side chain (carbon 4) by a sulfur or an oxygen decreases the susceptibility of the compound to the enzyme to a certain extent. Thialysine derivatives (# 161-168) are hydrolyzed almost as well as the corresponding lysine derivative, but oxalysine derivatives (# 148, 170, 171) are hydrolyzed much more slowly.

5) Several derivatives have been synthesized in which the number of methylene groups (n) in the side chain has been varied (# 152-160, 175-179). Derivatives of the higher homologue of arginine, homoarginine, are hydrolyzed by trypsin, but at a much slower rate than are the corresponding arginine derivatives. γ-Guanidino-α-aminobutyric acid derivatives (norarginine) (# 159, 160) are hydrolyzed very slowly by trypsin, as are the derivatives of ornithine (# 153-158). The action of trypsin on the derivatives of the higher homologues of lysine has not been studied.

6) Also hydrolyzed by trypsin are compounds which bear a structural relationship to a normal substrate (# 147-151). β-Alanyl-glycine methyl ester (# 147) is one such compound. Compounds which either contain or resemble lysine or arginine are known as specific substrates. There

are, however, a number of other compounds which are hydrolyzed by the enzyme. These are known as non-specific substrates. These compounds possess some of the features required of specific substrates, but lack others. For example, α-N-benzoylglycine ethyl ester, which has no side chain or ω-nitrogen, is hydrolyzed by trypsin, but at 1/100,000th the rate of a specific substrate. Also found in this category, as well as in the pseudo-substrates category, are compounds which, although resembling a specific substrate, have no positive charge on the side chain. These are known as neutral substrates (# 173, 174, 296). The rate of hydrolysis of these compounds is also very much slower than that of a specific substrate.

Object of Our Research

Thus the current state of our knowledge about trypsin contains several gaps. It was the aim of this project to attempt to clarify certain points about the specificity of the enzyme, particularly with respect to ε-N-substituted lysines.

1) Although there are in the literature some qualitative data to indicate that specific substrates with an alkyl residue on the ω-nitrogen are hydrolyzed, quantitative data are lacking. It was decided to investigate the quantitative as well as qualitative effect of such substituents upon the rate of hydrolysis. For this study

a series of ε-mono-, di- and trimethyl-lysines was prepared and studied. Also prepared was poly-ε-N-methyl-lysine.

2) It has been generally believed that substitution of the ε-amino group by an acyl moiety renders the substrate resistant to trypsin. However, there is some indication that this may not be true in all cases. Kloss and Schröder (50) showed that several formyl and tosyl peptides were slowly hydrolyzed by trypsin. In order to do this they used the conditions of Walton et al. (154). This involved dissolving the peptide in dimethylformamide and adding this to a stirred solution of the enzyme. We have attempted to verify this unexpected finding by preparing some less complex and more typical substrates bearing a formyl group on the ε-amino nitrogen.

3) The lack of any data on the higher homologues of lysine prompted an investigation of the effect of chain length for these substrates.

In general, the compounds synthesized were based on three model substrates: L-lysine ethyl ester, α-N-benzoyl-L-lysine methyl ester and α-N-benzoyl-L-lysinamide. This thesis describes the preparation of these compounds and the action of trypsin on them.

Preparation of Substrates

I. Materials

1. Reagents

The following reagents were purchased from the firms indicated.

Carbobenzoxy chloride (Nutritional Biochemicals, Pierce Chemical); Chloroauric acid (British Drug Houses); Diethyl acetamidomalonate (Pierce Chemical); Homopiperidine (Aldrich); L-Lysine·HCl (Nutritional Biochemicals); Ninhydrin (Pierce Chemical); L-Ornithine·HCl (General Biochemicals); Phosgene (Matheson); Potassium acid phthalate (Merck).

2. Purification of Reagents

a) Acetic Acid:

Commercial acetic acid was dried by cooling it and filtering off the crystalline compound in a cold room (155).

b) Benzaldehyde:

Commercial benzaldehyde was washed with 10% sodium carbonate and water, followed by drying (magnesium sulfate). The drying agent was filtered off and the benzaldehyde was distilled under nitrogen at reduced pressure. The fraction distilling at $79^{\circ}/25$ mm Hg was collected (156).

c) Dimethylformamide:

Commercial dimethylformamide was treated with solid potassium hydroxide, filtered, and treated with solid

calcium oxide. The product was distilled at atmospheric pressure and the fraction distilling at 152-153° was collected (157).

d)Ethanol:

Commercial ethanol was dried by refluxing it with magnesium and iodine for ½ hour followed by distillation with the careful exclusion of moisture. The fraction distilling at 78° was collected and kept in a tightly sealed bottle (158).

e)Ethyl Acetate:

Commercial ethyl acetate was purified by refluxing it with acetic anhydride and ethanol for four hours. This was followed by distillation at atmospheric pressure. The fraction distilling at 76-77° was collected and kept in a tightly stoppered bottle (159).

f)Methanol:

Commercial methanol was purified by refluxing it with iodine and magnesium for one hour, followed by distillation at atmospheric pressure. The fraction distilling at 65° was collected and kept in a tightly stoppered bottle (160).

g)Thionyl Chloride:

Reagent grade thionyl chloride was further purified by distilling it first from isoquinoline and then from raw linseed oil. The fraction distilling at 78-79° was collected (161).

3. Preparation of Reagents

a) Diazomethane in Ether:

This reagent was prepared as described by Vogel (162) using N-methyl,N-nitroso-p-toluenesulfonamide and alcoholic potassium hydroxide in ether. The product was distilled over with the ether. The yellow solution was kept at 4° until used.

b) p-Nitrophenyl Chloroacetate:

This compound was prepared as described by Benoiton (163) using p-nitrophenol sodium salt and chloroacetyl chloride. Yield: 70%, m.p. 94°, (lit. (163), 94°).

II. Methods

1. General Techniques

a) Infrared Spectra:

The infrared spectra were run on a Unicam spectrophotometer, model SP-200 (Unicam Instruments, Cambridge, England). The samples were scanned from 650 to 5000 cm^{-1} (2-15 microns). The instrument accepted liquid films as well as potassium bromide discs. The experimental section describes the position and intensity of the peaks between 1400 and 2000 cm^{-1} only. This is the region of most interest since it contains both the carbonyl and amide absorptions. The abbreviations used for the intensities are: vs, very strong; s, strong; m, medium; w, weak.

b)Melting Points:

Melting points were determined by the capillary method on a Unimelt apparatus (A.H. Thomas Co.), provided with a thermometer which was calibrated with known standards.

c)Elemental Analyses:

The elemental analyses reported were performed by Microanalytical Laboratories (C. Daessle, Montreal; and A. Gygli, Toronto).

d)Resins:

i) Dowex 50 x 8

This resin, 20-50 mesh, and 200-400 mesh, 1.9 meq/ml capacity was cleaned as follows: The resin was washed with water until it was color-throw free and stirred for 1 hour with 2N NaOH (5 bed volumes), then transferred to a fritted glass funnel and washed with distilled water until the filtrate was neutral to pH paper. The resin was stirred with 4N HCl (5 bed volumes) for several hours and filtered, then washed again with distilled water until the filtrate was neutral to pH paper.

ii) Amberlite IRC 50

This resin was washed with 1N HCl (3 bed volumes) and rinsed with distilled water while on the column until the effluent was free of chloride ions.

2. Thin Layer Chromatography

Ascending chromatography on cellulose covered plates was employed throughout. The plates were prepared by spraying a suspension of cellulose in water onto the appropriate piece of glass with a paint sprayer. The following solvent systems were used:

a) tert-Butanol:90% formic acid:water,
(70:15:15, v/v)

b) n-Butanol:acetic acid:water:pyridine,
(30:6:24:20, v/v)

The chromatograms were air dried and sprayed with a 0.5% solution of ninhydrin in butanol (Ninspray, Nutritional Biochemicals). Compounds with free amino groups gave characteristic blue spots upon warming the chromatograms.

3. Amino Acid Analysis

a) Operation

Amino acid analyses were carried out on a modified Beckman Amino Acid Analyzer (Model 120B) essentially according to the method of Spackman, Stein and Moore (164). The original instrument was equipped with two long (0.9 x 50 cm; numbered Ia and Ib) and one short (0.9 x 7 cm, numbered II) columns, maintained at 57° by a circulating water bath. An additional column of intermediate length (17 x 0.9 cm; numbered III) was constructed using Beckman

fittings and Aminex-5 resin, purchased from Bio-Rad (Richmond, California), which was essentially the same as the resin in column II.

Samples were applied to the column at pH 2.2 (0.1 to 2.0 ml) and rinsed in with the eluting buffer (3 x 0.2 ml). The amino acids were eluted with citrate buffers of appropriate pH (3.28, 4.25, 5.28). The effluent from the column was mixed continuously with ninhydrin reagent and passed through a boiling water bath to a colorimeter. The colorimeter was equipped with three photocells (570, 570 and 420 mμ) whose outputs were recorded on paper as three curves by a recorder. The chart speed was six inches per hour, and the peaks were printed as dots every five seconds.

The amount of each amino acid present was determined by estimating the area under the peak it produced. This was most conveniently done by measuring the height of the peak, subtracting the base line and multiplying by the width. The width was obtained by counting the number of dots above the half-height of the peak. A constant was determined by passing a sample of known amount through the column. The constant is given by:

$$\text{constant} = \frac{\text{height x width}}{\text{amount} \quad (\mu\text{moles})}$$

The constants given in Table II are the average values of several runs at different known concentrations. The reproducibility and error is ± 3% (165).

b) Analyses of Amino Acid Esters

A number of the amino acid esters which were synthesized were not crystalline. In these cases the non-crystalline esters were dried *in vacuo* (P_2O_5,NaOH) to constant weight, and were dissolved in water at the appropriate concentration for use in kinetic studies. A small sample (2 ml) was treated with either NaOH or HCl and run on a column of the amino acid analyzer. The amount of the component amino acid was then measured.

ε-N-Formyl-L-lysine methyl ester·HCl and ε-N, ε-N-dimethyl-L-lysine·ethyl ester·HCl were saponified with a 10% excess of 1N NaOH. The solutions were then brought to pH 2.2 and analyzed. The theoretical amount of the amino acid which should have been produced was calculated and compared with the experimental result. No adjustment was made to the concentration if the results were within ± 5%.

Similarly, the α-N-benzoyl amino acid ester solutions were hydrolyzed *in vacuo* with 6N HCl at 110° for 72 hours. The hydrolysis tubes (Precision Scientific) were frozen in liquid nitrogen prior to the evacuation.

TABLE II

Chromatographic Data from the Amino Acid Analyzer

Compound	Column	Constant	Time (min)	Buffer
L-Lysine	I	22.06	164.0	5.28
	II	23.80	25.0	5.28
ε-N-Methyl-L-lysine	I	20.15	178.0	5.28
	II	21.02	25.0	5.28
ε-N-Methyl,ε-N-carbobenzoxy-L-lysine	I	22.24	134.0	5.28
ε-N,ε-N-Dimethyl-L-lysine*	I	19.48	178.0	5.28
ε-N,ε-N-Trimethyl-L-lysine▲	I	18.48	159.0	5.28
α-N-Benzoyl-L-lysine	II	6.15	14.5	5.28
	III	6.15	31.5	5.28
ε-N-Formyl-L-lysine	I	18.82	99.0	3.28/4.25
DL-Homolysine	I	22.77	228.0	5.28
	II	22.77	33.0	5.28
ζ-N-Chloroacetyl-DL-homolysine	I	20.87	39.5	4.25
α-N-Benzoyl-DL-homolysine	II	4.26	16.0	5.28
	III	4.26	39.5	5.28
2,8-Diaminooctanoic Acid	II	--	113.5	5.28
L-Serine	III	21.10	21.0	3.28
ε-Amino-n-caproic Acid	I	6.16	128.0	5.28
	II	6.16	20.0	5.28

* α-N-Acetyl-ε-N,ε-N-dimethyl-L-lysine·$2H_2O$ (63.07 mg, 250 μmoles) was refluxed for 1.5 hours with 2N HCl (15 ml). The solution was evaporated and diluted to 100 ml. A five-fold dilution using 0.2N sodium citrate buffer pH 2.2 was made and the sample (1 ml) run on the analyzer.

▲ α-N-Benzoyl-ε-N,ε-N,ε-N-trimethyl-L-lysine gold salt (25.29 mg, 40 μmoles) was hydrolyzed *in vacuo* with 6N HCl (4 ml) at 110° for 72 hours. The solution was diluted to 25 ml with adjustment of the pH to 2.2. A sample (0.5 ml) was run on the analyzer.

The solutions were brought to pH 2.2 with the aid of 4N NaOH and diluted to a convenient volume. Samples were run on the analyzer as before. The results are given in the section on the individual compounds.

4. Determination of Dissociation Constants

We wished to determine the dissociation constants for the ω-amino groups of the α-N-benzoyl amino acid amides prepared. This was done by the method of Sorensen (166) as described in Chemistry of the Amino Acids (167). This corresponds to method E_3bg of the International Union of Pure and Applied Chemistry (168), and is described as approximate (± 0.04 pK unit). The method consisted of measuring the pH of the compound in the presence of a known amount of standard NaOH. This necessitated the use of a stable pH meter (Radiometer, Copenhagen; Model 22) and electrodes with a very low sodium error (Radiometer; K4112 calomel; G 2222B glass). The measurements were performed at 25° in the presence of 0.1M NaCl. The pH meter was standardized with at least two buffers and the readings verified between samples. The accuracy of the method was checked by using compounds having well determined pK values. Glycine and tyrosine ethyl ester were selected. The results for the determination of the pK_2 of glycine are given as an example.

0.1M NaOH ml	0.1M glycine ml	0.133M NaCl ml	pH	pOH	X_2	pK_2
0.100	0.150	0.75	9.97	4.03	0.660	9.68
0.075	0.175	0.75	9.60	4.40	0.429	9.72
0.050	0.200	0.75	9.30	4.70	0.250	9.78
0.025	0.225	0.75	8.87	5.13	0.111	9.79
0.020	0.230	0.75	8.75	5.25	0.087	9.77

$$pK_2 = 9.75 \pm 0.02 \text{ SE}$$

The 0.1N NaOH (BDH) was standardized using potassium acid phthalate and phenolphthalein. The water used for the determination was distilled three times in an all-glass apparatus.

The pK given for glycine in the literature is 9.78 (169). Similarly tyrosine ethyl ester was found to have a pK of 7.26. The literature value (170) is 7.33 at a lower ionic strength.

The calculations were done using the following equations:

$$pH + pOH = pKw = 14.00$$

and

$$pK_2 = pH - \log \frac{X_2}{1 - X_2} \quad \text{(Henderson-Hasselbach)}$$

where: $$X_2 = \frac{[NaOH] - [OH^-]}{[glycine]}$$

e.g. for the first sample.

$$pOH = 4.03$$

$$X_2 = \frac{0.0100N - [OH^-]}{0.0150N}$$

$$X_2 = 0.660$$

$$pK_2 = 9.97 - \log \left\{ \frac{0.660}{0.340} \right\}$$

The dissociation constants of the amides were determined in the same way and the results appear with the data on the corresponding compounds in the experimental section (pp 46, 53, 56, 59, 63, 73).

III. Syntheses

Throughout the experimental section the molecular weight of each compound appears in brackets after its name. An asterisk designates a new compound.

Synthetic work which has been previously published is not described. The synthesis of new compounds or compounds prepared by methods other than those in the literature is described in full. All compounds were dried *in vacuo* over phosphourus pentoxide and sodium hydroxide pellets unless otherwise stated. All evaporations were done *in vacuo* in a rotary evaporator (Buchi, Switzerland).

1. Ornithine Derivatives

L-Ornithine Ethyl Ester·2HCl (233.14)

This compound was prepared by the method of Brenner and Huber (171) using thionyl chloride.

To dry ethanol (25 ml) in an ice-bath was added purified thionyl chloride (1.6 ml, 22 mmoles) followed by L-ornithine·HCl (3.37 gm, 20 mmoles). The mixture was refluxed for one hour, at which point all the amino acid had dissolved. The solution was kept 24 hours at room temperature. The solvent was removed and the resulting oil was re-evaporated with ethanol until no further odor of thionyl chloride could be detected. The oil was crystallized from ethanol-ether and finally from ethanol.

Yield: 2.98 gm (64%), m.p. 173-175°

I.R. max: 1415 (m), 1450 (w), 1460 (w), 1505 (s), 1580 (w), 1600 (w), 1740 (vs).

α-N-Benzoyl-L-ornithine Methyl Ester·H_2O (268.31)

This compound was purchased from Cyclo Chemical Corp., Los Angeles, California. Cat. No. 3820-1. The compound was sold as being chromatographically homogeneous in phenol:water (100:20 v/v); n-butanol: acetic acid: water (450:50:125, v/v) and 2-butanol: 3.3% ammonia (150:60, v/v). Found: C, 57.6; H, 8.1; N, 11.0. $C_{13}H_{20}N_2O_4$ requires C, 58.2; H, 7.5; N, 10.4.

2. Lysine Substrates

a) Non-Alkylated

L-Lysine Ethyl Ester·2HCl (247.17)

This compound was prepared by the method of Brenner and Huber (171), using L-lysine·HCl and thionyl chloride. Yield: 46%, m.p. 143.5-145° (lit. (172) 143.5-144.5°). I.R. max: 1405(w), 1500(s), 1600(s), 1735(s).

ε-N-Carbobenzoxy-L-lysine (280.32)

This compound was prepared as described by Neuberger and Sanger (173) using the copper salt of lysine and carbobenzoxy chloride under Schotten-Baumann conditions. The copper was removed with hydrogen sulfide, or by the method of Zahn (174) using potassium cyanide. Yields were from 70 to 75%, m.p. 257-258° (lit. (172) 255°). I.R. max: 1420(m), 1450(w), 1540(s), 1580(s), 1600(m), 1680(vs).

α-N,ε-N-Dicarbobenzoxy-L-lysine (414.15)

This compound was prepared as described by Bergmann, Zervas and Ross (175), from the amino acid and carbobenzoxy chloride under Schotten-Baumann conditions. Yield: 98%. The compound crystallized after standing for several weeks. I.R. max: 1405(w), 1460(w), 1540(s), 1650(w), 1680(s), 1705(s).

α-N-Benzoyl-ε-N-carbobenzoxy-L-lysine (384.42)

This compound was prepared as described by Ross and Green (176), using ε-N-carbobenzoxy-L-lysine and benzoyl chloride under Schotten-Baumann conditions. Yields were from 46 to 85%, m.p. 109-111° (lit. (176) 107°).
I.R. max: 1550(s), 1650(s), 1690(s), 1710(s).

α-N-Benzoyl-L-lysine (250.29)

This compound was prepared as described by Ross and Green (176) by hydrogenation of α-N-carbobenzoxy-L-lysine in water-acetic acid. Yields were from 48 to 87%, m.p. 250° (lit. (176) 250°). The product was found to contain 0 to 0.9 molar % lysine as contaminant.
I.R. max: 1400(m), 1490(w), 1580(s), 1630(s).

α-N-Benzoyl-ε-N-carbobenzoxy-L-lysine Methyl Ester (398.45)

α-N-Benzoyl-ε-N-carbobenzoxy-L-lysine (4.66 gm, 12 mmoles) was dissolved in absolute methanol (50 ml) and a solution of diazomethane in ether was added until a persistent yellow color was obtained. A drop of glacial acetic acid was added to destroy any residual diazomethane, and the solution was evaporated. The resulting oil was re-evaporated several times with methanol yielding a white gum which solidified on standing several days.
Yield: 4.34 gm (98%).
I.R. max: 1450(m), 1540(s), 1640(s), 1690(s), 1710(s), 1735(s).

α-N-Benzoyl-ε-N-carbobenzoxy-L-lysinamide (383.43)

This compound was prepared as described by Bergmann and Hofmann (177), using α-N-benzoyl-ε-N-carbobenzoxy-L-lysine methyl ester and methanol saturated with ammonia. Yields were from 45 to 71%, m.p. 172-173° (lit. (177) 172-173°). Found: C, 65.9; H, 6.4; N, 11.4. $C_{21}H_{25}N_3O_4$ requires C, 65.8; H, 6.6; N, 11.0.
I.R. max: 1540(s), 1640(s), 1660(vs), 1690(vs).

α-N-Benzoyl-L-lysinamide·HCl (285.77)

This compound was prepared as described by Bergmann and Hofmann (177), by hydrogenation of α-N-benzoyl-ε-N-carbobenzoxy-L-lysinamide in methanol and 1N HCl. Yields were from 80-87%, m.p. 202-203° (lit. (177) 200-201°). Found: C, 56.1; H, 7.3; N, 13.7. $C_{19}H_{20}ClN_3O_2$ requires C, 56.1; H, 7.4; N, 14.0.
pK 9.69 ± 0.04 S.E.
I.R. max: 1500(w), 1540(s), 1610(s), 1650(vs), 1680(s).

α-N-Benzoyl-L-lysine Methyl Ester·HCl (300.78)

a) This compound was prepared as described by Elmore, Roberts and Smyth (31), by hydrogenation of α-N-benzoyl-ε-N-carbobenzoxy-L-lysine methyl ester using 1N HCl instead of p-toluenesulfonic acid. Yield: 97%, oil.

b) The compound was also prepared using thionyl chloride and α-N-benzoyl-L-lysine.

α-N-Benzoyl-L-lysine (2.50 gm, 10 mmoles) was added to a solution of purified thionyl chloride (0.8 ml, 11 mmoles) in cold absolute methanol (50 ml). The solution was refluxed for 1 hour and kept 24 hours at room temperature. The solvent was evaporated and the resulting oil re-evaporated with methanol until no odor of thionyl chloride could be detected. The oil was found to contain from 0.90 to 1.82 molar % α-N-benzoyl-L-lysine. The infrared spectrum of the compound was identical to that of the compound prepared by the method of Elmore et al. The ester was purified for kinetic work by dissolving it in water, adding solid anhydrous potassium carbonate and extracting with ether. The ester was obtained as the hydrochloride by bubbling dry HCl gas through the ethereal solution. The resulting oil was obtained as a hygroscopic foam by repeated evaporation from ether. Yields were from 34 to 61%. A sample was hydrolyzed as previously described. Found: 1.70 μmoles; requires 1.64 μmoles lysine.

I.R. max: 1450(m), 1495(s), 1575(m), 1600(m), 1640(s), 1735(s).

b) Monomethyl

ε-N-Benzylidine-L-lysine (234.29)

This compound was prepared as described by Bezas and Zervas (178), using L-lysine HCl and freshly

distilled benzaldehyde in the presence of 2N LiOH. Yields were from 75 to 85%, m.p. 206-208°(dec.), (lit. (178) 206-208° (dec.)).

I.R. max: 1410(s), 1455(m), 1520(s), 1590(vs), 1605(s), 1640(s).

α-N-Carbobenzoxy-L-lysine (280.32)

This compound was prepared as described by Bezas and Zervas (178), using ε-N-benzylidine-L-lysine and carbobenzoxy chloride under Schotten-Baumann conditions. Yields were from 22 to 60%,m.p. 232-233° (dec.), (lit. (178) 232-233° (dec.)).

I.R. max: 1410(s), 1530(s), 1570(vs), 1620(w), 1660(m), 1710(vs).

α-N-Carbobenzoxy-ε-N-benzyl-L-lysine (370.44)

This compound was prepared as described by Benoiton (179), using borohydride reduction of the Schiff base formed between α-N-carbobenzoxy-L-lysine and freshly distilled benzaldehyde. Yields were from 38 to 76%, m.p. 209° (lit. (179) 210°)

I.R. max: 1410(s), 1470(m), 1510(s), 1590(s), 1710(s).

ε-N-Methyl-L-lysine·HCl (196.68)

This compound was prepared as described by Benoiton (179), using α-N-carbobenzoxy-ε-N-benzyl-L-lysine and a mixture of formaldehyde-formic acid followed by hydrogenation.

Yields were from 48 to 61%, m.p. 238-240° (dec.), (lit. (179) 237-239° (dec.)). The product contains no lysine contaminant.

I.R. max: 1420(m), 1440(m), 1520(m), 1570(s), 1610(s), 1640(s).

ε-N-Methyl-L-lysine Ethyl Ester·2HCl (261.20)

This compound was prepared using thionyl chloride and ε-N-methyl-L-lysine.

ε-N-Methyl-L-lysine·HCl (0.98 gm, 5 mmoles) was added to ice cold ethanol (15 ml) containing purified thionyl chloride (0.36 ml, 5.5 mmoles). The solution was refluxed for 1 hour and kept 24 hours at room temperature. The solvent was evaporated and the resulting oil re-evaporated with ethanol until no odor of thionyl chloride was detected. The oil was crystallized several times from ethanol-ether. Yield: 0.50 gm (38%), m.p. 145-146°, (lit. (180) 145°). The yields from other preparations were from 38 to 71%.

I.R. max: 1465(m), 1505(m), 1585(m), 1605(w), 1735(vs).

ε-N-Methyl, ε-N-carbobenzoxy-L-lysine (294.35)

This compound was prepared as described by Benoiton and Deneault (26), using the copper salt of ε-N-methyl-L-lysine and carbobenzoxy chloride. Yields were from 16 to 82%, m.p. 231-233°, (lit. (26) 210-212°).

I.R. max: 1410(s), 1520(s), 1590(s), 1615(s), 1700(vs).

α-N-Benzoyl-ε-N-methyl,ε-N-carbobenzoxy-L-lysine (398.45)

This compound was prepared as described by Benoiton and Deneault (26), using ε-N-methyl,ε-N-carbobenzoxy-L-lysine and benzoyl chloride under Schotten-Baumann conditions. Yields were from 98 to 99%. The compound was an oil.

I.R. max: 1405(m), 1455(m), 1490(m), 1535(m), 1575(w), 1640(s), 1660(s), 1685(s), 1700(s), 1715(s).

α-N-Benzoyl-ε-N-methyl,ε-N-carbobenzoxy-L-lysine Methyl Ester* (412.48)

α-N-Benzoyl-ε-N-methyl,ε-N-carbobenzoxy-L-lysine (1.79 gm, 4.5 mmoles) was dissolved in absolute methanol (25 ml) and a solution of diazomethane in ether was added until a yellow color persisted. A few drops of acetic acid were added to destroy the residual diazomethane and the solution was evaporated yielding a clear oil. This was re-evaporated several times with methanol.

Yield: 1.80 gm (99%). The compound was an oil.

I.R. max: 1405(m), 1455(m), 1490(m), 1575(w), 1600(w), 1655(s), 1690(s), 1735(s).

α-N-Benzoyl-ε-N-methyl-L-lysine Methyl Ester* (278.35)

α-N-Benzoyl-ε-N-methyl,ε-N-carbobenzoxy-L-lysine methyl ester (1.80 gm, 4.3 mmoles) was dissolved in a mixture of ethanol, methanol and acetic acid (2:4:1, v/v;

70 ml) and hydrogenated with 10% palladium on charcoal (1 gm). The hydrogen was replaced frequently; when consumption thereof had ceased (24 hr), the mixture was filtered through celite and evaporated, yielding a clear oil. Yield: 1.20 gm (99%). The compound was an oil and gave only one spot with ninhydrin in butanol: acetic acid: water (160:15:20, v/v).

I.R. max: 1415(m), 1495(w), 1540(s), 1555(s), 1570(w), 1600(w), 1640(s), 1705(m), 1720(s), 1735(s).

α-N-Benzoyl-ε-N-methyl-L-lysine* (264.32)

α-N-Benzoyl-ε-N-methyl,ε-N-carbobenzoxy-L-lysine (1.50 gm, 3.1 mmoles) was dissolved in 90% acetic acid (50 ml) and hydrogenated with 10% palladium on charcoal (0.5 gm). The hydrogen was replaced frequently; when consumption thereof had ceased (24 hr), the solution was filtered through celite and evaporated. The residual oil was triturated in acetone giving a hygroscopic solid. Yield: 0.76 gm (76%). A sample was hydrolyzed as described previously and run on the amino acid analyzer. Found: 50.3 μmole; requires 51.9 μmoles ε-N-methyl-L-lysine.

I.R. max: 1400(s), 1490(w), 1540(s), 1585(s), 1630(s), 1645(s).

α-N-Benzoyl-ε-N-methyl-L-lysine Methyl Ester·HCl* (314.81)

This compound was prepared using thionyl chloride.

α-N-Benzoyl-ε-N-methyl-L-lysine (0.75 gm; 2.8 mmoles) was added to ice-cold absolute methanol (25 ml) containing purified thionyl chloride (0.22 ml, 3.0 mmole). The solution was refluxed for 1 hour and kept overnight at room temperature. The solvents were evaporated and the resulting oil was re-evaporated with methanol until no odor of thionyl chloride remained. Yield: 0.77 gm (86%). A sample was hydrolyzed as previously described. Found: 18.2 μmoles; requires 20.0 μmoles ε-N-methyl-L-lysine.

I.R. max: 1435(m), 1445(m), 1455(m), 1470(m), 1490(m), 1515(m), 1535(m), 1550(m), 1570(w), 1600(w), 1625(m), 1640(m), 1650(m), 1735(m).

α-N-Benzoyl-ε-N-methyl-L-lysinamide·HCl (299.80)

α-N-Benzoyl-ε-N-methyl-L-lysine methyl ester (0.60 gm, 2.1 mmoles) was dissolved in methanol saturated with ammonia. The solution was kept at room temperature in a stoppered flask for 48 hours. At the end of this period the solution was evaporated and 1N HCl (10 ml) was added. The mixture was evaporated repeatedly, yielding a beige-colored oil. The product was crystallized from ethanol. Yield: 0.10 gm (15%). Yields from other preparations were from 14 to 27%, m.p. 199-200° (lit. (26) 195-197°). Found: C, 56.1; H, 7.3; N, 13.7. $C_{14}H_{22}ClN_3O_2$ requires C, 56.1; H, 7.4; N, 14.0.

pK 10.41 ± 0.09 S.E.

I.R. max: 1550(m), 1575(m), 1600(w), 1640(s), 1680(m).

c) Dimethyl

α-N-Acetyl-ε-N,ε-N-dimethyl-L-lysine·$2H_2O$ (252.32)

This compound was prepared as described by Benoiton (179), using α-N-acetyl-L-lysine and formaldehyde. Yield: 57%, m.p. 192-194° (lit. (179) 196-198°).

I.R. max: 1440(w), 1460(m), 1570(vs), 1600(vs), 1630(vs).

ε-N,ε-N-Dimethyl-L-lysine·HCl (210.71)

This compound was prepared as described by Benoiton (179), by mild acid hydrolysis of α-N-acetyl-ε-N,ε-N-dimethyl-L-lysine·$2H_2O$. Yield: 62%, m.p. 143-144°, (lit. (179) 145°)

ε-N,ε-N-Dimethyl-L-lysine Ethyl Ester·2HCl* (261.20)

ε-N,ε-N-Dimethyl-L-lysine·HCl (2.11 gm, 10 mmoles) was added to ice-cold dry ethanol (25 ml) containing purified thionyl chloride (0.89 ml, 11 mmoles). The mixture was refluxed for one hour and kept 24 hours at room temperature. The solvent was removed, and the oil re-evaporated with ethanol until no odor of thionyl chloride was detected. The oil could not be crystallized. A sample was saponified as previously described and run on the amino acid analyzer. Found: 9.16 μmoles; requires

10.0 μmoles ε-N,ε-N-dimethyl-L-lysine. There was 0.55 μmole lysine as contaminant.

I.R. max: 1450(w), 1470(w), 1490(w), 1505(w), 1550(w), 1600(w), 1630(w), 1640(w), 1660(w), 1735(s).

α-N-Benzoyl-ε-N,ε-N-dimethyl-L-lysine (278.35)

This compound was prepared as described by Benoiton (179), by treatment of α-N-benzoyl-L-lysine with formaldehyde, followed by hydrogenation. Yields were from 26-64%, m.p. 208°, (lit. (179) 209°).

I.R. max: 1400(s), 1455(w), 1510(s), 1600(s), 1625(s), 1660(s).

α-N-Benzoyl-ε-N,ε-N-dimethyl-L-lysine Methyl Ester* (292.38)

α-N-Benzoyl-ε-N,ε-N-dimethyl-L-lysine (1.75 gm, 6.3 mmoles) was dissolved in absolute methanol (50 ml) and cooled in an ice-bath. A solution of diazomethane in ether was added until a faint yellow color persisted. One drop of glacial acetic acid was added to destroy the excess diazomethane. Evaporation of the solvent yielded a clear oil which could not be crystallized. Yield: 1.81 gm (99%).

I.R. max: 1400(m), 1495(s), 1545(s), 1590(w), 1610(s), 1645(s), 1740(s).

α-N-Benzoyl-ε-N,ε-N-dimethyl-L-lysinamide HCl·H_2O* (331.84)

α-N-Benzoyl-ε-N,ε-N-dimethyl-L-lysine methyl ester (0.73 gm, 2.5 mmoles) was dissolved in methanol saturated with ammonia. The solution was kept for several days at room temperature in a stoppered flask. Evaporation of the solvent gave a product which was contaminated with ester. The oil was reamidated, and evaporation yielded an oil which was treated with 1N HCl (10 ml) and re-evaporated, yielding a white powder. This was re-crystallized from ethanol-acetone. Yield: 0.60 gm (80%), m.p. 155-157°. The compound was hygroscopic. Found: C, 54.2; H, 7.8; N, 12.7. $C_{15}H_{24}ClN_3O_2 \cdot H_2O$ requires C, 54.5; H, 7.6; N, 12.7. Weight loss at 100° (P_2O_5): 5.0%; requires 5.4%. pK 9.53 ± 0.01 S.E. I.R. max: 1420(w), 1500(m), 1540(m), 1585(w), 1650(s), 1680(s).

α-N-Benzoyl-ε-N,ε-N-dimethyl-L-lysine Methyl Ester·HCl* (328.84)

This compound was prepared using α-N-benzoyl-ε-N, ε-N-dimethyl-L-lysine and thionyl chloride.

α-N-Benzoyl-ε-N,ε-N-dimethyl-L-lysine (0.75 gm, 2.6 mmoles) was added to ice-cold absolute methanol (40 ml) containing purified thionyl chloride (0.20 ml,

3.0 mmoles). The solution was refluxed for one hour and kept for 24 hours at room temperature. Evaporation of the solvent yielded an oil which was re-evaporated several times with ethanol until no odor of thionyl chloride was detected. The oil could not be crystallized. A small aliquot was hydrolyzed as previously described, and run on the amino acid analyzer. Found: 24.6 μmoles; requires 24.0 μmoles ε-N,ε-N-dimethyl-L-lysine.

d) Trimethyl

α-N-Benzoyl-ε-N,ε-N-trimethyl-L-lysine* (292.38)

This compound was prepared by the method of Enger and Halle (180), using dimethyl sulfate and α-N-benzoyl-L-lysine.

α-N-Benzoyl-L-lysine (1.45 gm, 5.7 mmoles) was added to a mixture of barium hydroxide·$8H_2O$ (16 gm, 50 mmoles) and water (20 ml). Dimethyl sulfate (6.0 ml, 6.4 mmoles) was then added, and the mixture was stirred for three days in a fume cupboard. An aliquot was run on column II of the amino acid analyzer, and was shown to be free of any unreacted starting material. The mixture was then brought to pH 2.0 (Congo red) with 6N H_2SO_4 and the precipitated barium sulfate removed by contrifugation. The supernatant liquid was stirred for several hours with Dowex 50 resin

(100 ml, H^+ form). At the end of this period the resin was filtered off, washed with a large volume of water and suspended in 4N NH_4OH (200 ml) for three hours. The resin was filtered off and the aqueous solution evaporated, giving a yellow oil. This was crystallized from ethanol-acetone. Yield: 1.12 gm (67%), m.p. 240-241° (dec.). A gold salt was prepared by dissolving chloroauric acid (0.5 gm) in concentrated HCl (0.25 ml) and diluting with water (3.0 ml). This solution was added to an aqueous solution of the betaine (100 mg/ml) and left in a refrigerator. The yellow crystals produced were collected and recrystallized from 0.01% HCl, m.p. 159-161°. Found: C, 30.9, 30.6; H, 4.0,4.2; N, 4.7,4.3; Au, 31.2,31.5. $C_{16}H_{25}N_2O_3 \cdot AuCl_4$ requires C, 30.4; H, 4.0; N, 4.4; Au, 31.2.
I.R. max: 1400(s), 1420(w), 1450(w), 1620(s), 1650(s).

<u>α-N-Benzoyl-ε-N,ε-N,ε-N-trimethyl-L-lysine Methyl Ester·Cl^{-*} (340.86)</u>

α-N-Benzoyl-ε-N,ε-N,ε-N-trimethyl-L-lysine (1.0 gm, 3.4 mmoles) was added to ice-cold absolute methanol (25 ml) containing purified thionyl chloride (0.30 ml, 4.0 mmoles). The solution was refluxed for 1 hour and kept 24 hours at room temperature. The solvent was removed yielding a clear oil. This was re-evaporated several times with methanol until no odor of thionyl chloride could be detected. The

oil could not be crystallized. Yield: 0.98 gm (81%). An aliquot was hydrolyzed as previously described and run on the amino acid analyzer. Found: 21.8 μmoles; requires 20.0 μmoles ε-N,ε-N,ε-N-trimethyl-L-lysine.
I.R. max: 1460(w), 1495(s), 1540(s), 1650(s), 1735(s).

α-N-Benzoyl-ε-N,ε-N,ε-N-trimethyl-L-lysinamide·Cl^{-*} (327.85)

α-N-Benzoyl-ε-N,ε-N,ε-N-trimethyl-L-lysine methyl ester·Cl^- (0.98 gm, 2.8 mmoles) was dissolved in methanol saturated with ammonia (100 ml). The solution was kept several days at room temperature in a stoppered flask. Evaporation yielded a beige-colored powder which was recrystallized from ethanol. Yield: 0.90 gm (81%), m.p. 248-250°. The compound was hygroscopic and was dried before analysis. Found: C, 58.4; H, 8.3; N, 12.7; Cl, 10.8. $C_{16}H_{26}ClN_3O_2$ requires C, 58.6; H, 8.0; N, 12.8; Cl, 10.8. No pK.
I.R. max: 1420(w), 1495(m), 1540(s), 1580(w), 1650(s), 1690(m).

e) Acylated

ε-N-Formyl-L-lysine (174.20)

This compound was prepared as described by Hofmann et al. (181), using the copper salt of lysine and ethyl formate at pH 10.0, with the exception that the pH was

maintained by a pH-stat (Radiometer). The product was invariably contaminated with lysine (2 to 8 molar %). This was removed by passage through Amberlite IRC-50 as described by Okawa and Hase (182). After several passes through the resin the yields were from 12 to 27%. The final product contained 0 to 0.5 molar % lysine, m.p. 229-230°, (lit. (181) 214-215°).

I.R. max: 1420(m), 1465(w), 1530(s), 1590(s), 1620(w), 1660(s).

α-N-Carbobenzoxy-ε-N-formyl-L-lysine (308.33)

a) This compound was prepared as described by Hofmann et al. (181), using ε-N-formyl-L-lysine and carbobenzoxy chloride under Schotten-Baumann conditions. Yield: 88%, m.p. 93-94° (lit. (181) 94-95°).

b) The compound was also prepared by formylation of α-N-carbobenzoxy-L-lysine using ethyl formate at pH 10.0.

α-N-Carbobenzoxy-L-lysine (2.80 gm, 10 mmoles) was dissolved in a mixture of 1N NaOH (35 ml) and methanol (35 ml). The temperature of the mixture was lowered to 5° and ethyl formate (20 ml, 250 mmoles) was added. The reaction was stirred continuously and the pH was maintained at 10.0 with a pH-stat (Radiometer) charged with 2N NaOH. After three hours the organic solvents were evaporated and the aqueous portion acidified to pH 2.0 (Congo red) with 2N HCl. The product was extracted into ethyl

acetate and washed in the presence of crushed ice with 1N HCl. This was followed by washings with 2% $NaHCO_3$ and water. The organic layer was dried (Na_2SO_4), filtered and evaporated, yielding a clear oil which crystallized after several hours standing. Yields were from 36 to 53%, m.p. 94-95° (lit. (181) 94-95°).

I.R. max: 1460(m), 1540(s), 1645(m), 1665(s), 1680(s), 1695(s), 1710(s), 1720(s).

α-N-Carbobenzoxy-ε-N-formyl-L-lysine Methyl Ester (322.36)

This compound was prepared as described by Hofmann et al. (181), using α-N-carbobenzoxy-ε-N-formyl-L-lysine and diazomethane. Yield: 99%, oil (lit. (182) oil).

I.R. max: 1445(m), 1455(m), 1535(m), 1660(s), 1680(s), 1700(s), 1715(s), 1735(s).

ε-N-Formyl-L-lysine Methyl Ester·HCl (224.69)

This compound was prepared as described by Hofmann et al. (181) by hydrogenation of α-N-carbobenzoxy-ε-N-formyl-L-lysine methyl ester in aqueous formic acid. This was followed by lyophilization in the presence of 1N HCl. Yield: 63%, oil (lit. (181) oil). A sample was saponified as previously described and run on the amino acid analyzer. The compound was contaminated with 6.68 molar % lysine.

I.R. max: 1450(m), 1460(m), 1540(m), 1560(m), 1650(m), 1660(s), 1680(s), 1690(m), 1735(s).

α-N-Benzoyl-ε-N-formyl-L-lysine* (278.30)

ε-N-Formyl-L-lysine (1.44 gm, 8.2 mmoles) was added to 1N NaOH (12 ml) in an ice bath. Benzoyl chloride (1.1 ml, 9.1 mmoles) and 1N NaOH (12 ml) were added in three equal but alternate portions over 30 minutes with vigorous mechanical stirring. The mixture was stirred an additional two hours at room temperature, extracted with ether (2 x 25 ml) and acidified (6N HCl) to pH 2.0 (Congo red). The gum which precipitated was extracted into ethyl acetate (3 x 50 ml) and washed with 1N HCl, 2% $NaHCO_3$ and water. The organic layer was dried (Na_2SO_4) and evaporated, yielding a clear oil which solidified on standing. Yield: 1.94 gm (85%), m.p. 84-86°.
I.R. max: 1410(m), 1495(m), 1550(s), 1585(s), 1640(s).

α-N-Benzoyl-ε-N-formyl-L-lysine Methyl Ester* (292.33)

α-N-Benzoyl-ε-N-formyl-L-lysine (1.94 gm, 6.9 mmoles) was dissolved in absolute methanol and cooled in an ice-bath. A solution of diazomethane in ether was added until a yellow color persisted. A drop of glacial acetic acid was added to destroy the excess diazomethane. The solution was evaporated yielding a clear oil. Yield: 1.90 gm (95%). Other preparations gave yields from 90 to 98%.

I.R. max: 1460(m), 1500(m), 1540(s), 1580(w), 1610(w), 1645(s), 1665(s), 1740(s).

α-N-Benzoyl-ε-N-formyl-L-lysinamide* (277.31)

α-N-Benzoyl-ε-N-formyl-L-lysine methyl ester (1.45 gm, 5.0 mmoles) was dissolved in methanol saturated with ammonia (100 ml). This was kept for two days at room temperature in a stoppered flask. Evaporation of the solution yielded an oil which solidified on cooling. The product was recrystallized from a small volume of ethanol. Yield: 0.43 gm (30%), m.p. 161-162°. Found: C, 61.2; H, 7.0; N, 14.8. $C_{14}H_{19}N_3O_2$ requires C, 60.7; H, 6.9; N, 15.1. No pK.
I.R. max: 1500(w), 1545(s), 1580(w), 1645(s), 1680(s).

α-N-Acetyl-L-lysine (188.23)

This compound was prepared as described by Neuberger and Sanger (173), using acetic anhydride and ε-N-carbobenzoxy-L-lysine followed by hydrogenation. Yield: 65%, m.p. 250° (dec.) (lit. (173) 250° (dec.)). The compound was contaminated with 0.8 molar % lysine.
I.R. max: 1400(m), 1440(m), 1580(vs), 1625(s), 1660(s).

ε-N-Acetyl-L-lysine (188.23)

This compound was prepared as described by Benoiton and Leclerc (183), using p-nitrophenyl acetate and the copper salt of lysine. Yields were 33 to 65%, m.p. 261-

262° (dec.) (lit. (174) 245-253° (dec.)).

I.R. max: 1415(s), 1445(w), 1475(w), 1530(vs), 1580(vs), 1640(vs).

3. Homolysine and Derivatives

Benzoyl Piperidine (189.25)

This compound was prepared as described by Vogel (184), using benzoyl chloride and piperidine under Schotten-Baumann conditions. Yields were from 78-84%, b.p. 145-146° (12.3 mm Hg), m.p. 50° (lit. (184) b.p. 184-186° (15 mm Hg), m.p. 46°).

I.R. max: 1440(vs), 1470(s), 1495(m), 1575(w), 1630(vs).

5-Benzamido-1-chloropentane (225.72)

This compound was prepared as described by von Braun (185), using benzoyl piperidine and phosphorus pentachloride, but the material was not vacuum distilled. Yields were from 17-54%, m.p. 58° (lit. (185) 66°).

I.R. max: 1460(w), 1490(m), 1540(s), 1590(m), 1610(w), 1640(s), 1710(w).

2,7-Diaminoheptanoic acid·HCl (Homolysine) (196.68)

This compound was prepared as described by Wada (186), using 5-benzamido-1-chloropentane and diethyl acetamidomalonate in sodium ethoxide. This was followed by alkaline and acid hydrolysis. Yields were from 14 to

26%, m.p. 265° (dec. (lit. (186) 271° (dec.)).
I.R. max: 1420(w), 1470(m), 1490(s), 1505(s), 1575(s), 1630(s).

DL-Homolysine Ethyl Ester·2HCl* (261.20)

This compound was prepared using the amino acid and thionyl chloride.

DL-Homolysine·HCl (1.98 gm, 10 mmoles) was added to absolute ethanol (25 ml) containing purified thionyl chloride (0.80 ml, 11 mmoles). The mixture was refluxed until all the amino acid dissolved (2 hours) and was kept for 24 hours at room temperature. The solution was evaporated yielding an oil. Ethanol was added and the solution was re-evaporated until no further odor of thionyl chloride was detected. The residue was recrystallized from ethanol-ether. Yield: 1.74 gm (66%), m.p. 157-159°. Found: C, 41.3; H, 8.6; N, 10.5.
$C_9H_{22}Cl_2N_2O_2$ requires C, 41.4; H, 8.5; N, 10.7.
I.R. max: 1420(m), 1475(w), 1500(s), 1600(s), 1735(vs).

ε-N-Acetyl-DL-homolysine* (202.25)

This compound was prepared by the method of Benoiton and Leclerc (183), using the copper salt of the amino acid and p-nitrophenyl acetate.

DL-Homolysine·HCl (0.49 gm, 2.5 mmoles) was dissolved in water and the solution brought to a boil. Excess basic

copper carbonate was added and the mixture boiled gently for $\frac{1}{2}$ hour. After cooling, the mixture was filtered through celite, and sodium bicarbonate (0.63 gm, 7.5 mmoles) was added. This was followed by p-nitrophenyl acetate (0.91 gm, 5.0 mmoles) and the reaction was stirred vigorously for 18 hours. The copper complex of ζ-N-acetyl-DL-homolysine was filtered off, washed with ethyl acetate and water and dried (P_2O_5). The copper was removed by suspending the compound in water-acetic acid containing charcoal and celite, and bubbling H_2S through the mixture. After $\frac{1}{2}$ hour the solution was freed of H_2S by bubbling air through it. The mixture was filtered through celite and evaporated, yielding a white solid. This was recrystallized from water-ethanol. Yield: 0.32 gm (60%), m.p. 261-262° (dec.). Found: C, 53.3; H, 9.1; N, 13.6. $C_9H_{22}N_2O_3$ requires C, 53.4; H, 9.0; N, 13.9. I.R. max: 1420(s), 1510(w), 1580(vs), 1640(vs).

ζ-N-Chloroacetyl-DL-homolysine* (236.71)

This compound was prepared by the method of Benoiton and Leclerc (183), using the copper complex of the amino acid and p-nitrophenyl chloroacetate exactly as described for the acetyl compound above. Yield: 0.54 gm (68%), m.p. 197-198°. Found: C, 45.4; H, 7.4; N, 11.7; Cl, 14.5. $C_9H_{17}ClN_2O_3$ requires C, 45.7; H, 7.2; N, 11.8; Cl, 15.0.

α-N,ζ-N,-Dichloroacetyl-DL-homolysine* (313.20)

This compound was prepared by the method of Greenstein and Winitz (187), using the amino acid and chloroacetyl chloride under Schotten-Baumann conditions.

DL-Homolysine·HCl (1.52 gm, 7.7 mmoles) was dissolved in 1N NaOH (7.7 ml) in an ice-bath. To this was added, in equal but alternate portions, 1N NaOH (19 ml) and chloroacetyl chloride (1.9 ml, 17.5 mmoles) over ½ hour with vigorous stirring. The mixture was then acidified by adding 2N H_2SO_4 (14 ml) and evaporated. The residue was extracted with boiling acetone (3 x 25 ml) and filtered. Evaporation of the acetone yielded a yellow oil. Yield: 1.73 gm (71%).

ζ-N-Carbobenzoxy-DL-homolysine* (294.35)

This compound was prepared by the method of Neuberger and Sanger (173), using the copper complex of the amino acid and carbobenzoxy chloride under Schotten-Baumann conditions.

DL-Homolysine (2.44 gm, 12.4 mmoles) was dissolved in water and the solution brought to a boil. Basic copper carbonate was added carefully until no more would dissolve and the boiling was continued for ½ hour. After cooling the mixture was filtered and 1N NaOH (13 ml) was added. The solution was cooled in an ice-bath and 1N NaOH (20 ml) and carbobenzoxy chloride (2.0 ml, 13.6 mmoles) were added

in equal but alternate portions over ½ hour with vigorous stirring. The mixture was stirred an additional 2 hours at room temperature. The copper complex of ζ-N-carbobenxoxy-DL-homolysine was filtered off and washed with water. The copper was removed by the method of Zahn (174). The compound was suspended in water and KCN (2 gm) was added. The mixture was stirred for 2 hours and then glacial acetic acid (2 ml) was added to destroy the residual KCN. The mixture was evaporated and the residue treated with boiling water. After cooling, the product was filtered off and recrystallized from water. Yield: 2.44 gm (66%), m.p. 251-252°. Other preparations gave yields from 38 to 66%. Found: C, 61.3; H, 7.6; N, 9.7. $C_{15}H_{22}N_2O_4$ requires C, 61.2; H, 7.5; N, 9.5.

I.R. max: 1420(s), 1460(w), 1550(m), 1580(s), 1620(m), 1680(s).

α-N-Chloroacetyl-ζ-N-carbobenzoxy-DL-homolysine* (370.84)

This compound was prepared by the method of Greenstein and Winitz (187) using ζ-N-carbobenzoxy-DL-homolysine and chloroacetyl chloride under Schotten-Baumann conditions.

ζ-N-Carbobenzoxy-DL-homolysine (1.25 gm, 4.2 mmoles) was dissolved in 1N NaOH (10 ml) in an ice-bath. This was followed by chloroacetyl chloride (0.37 ml, 4.7 mmoles) and the solution was stirred for several hours at room temperature. The mixture was acidified with 6N HCl to

pH 2.0 (Congo red), and the gum which precipitated was extracted into ethyl acetate (3 x 25 ml). The compound was extracted into 2% $NaHCO_3$ and this layer reacidified as above. Re-extraction into ethyl acetate was followed by drying (Na_2SO_4). The solution was filtered and evaporated, yielding an oil. Yield: 0.94 gm (60%).

α-N-Benzoyl-ζ-N-carbobenzoxy-DL-homolysine* (398.45)

This compound was prepared by the method of Ross and Green (176) for the corresponding lysine compound.

ζ-N-Carbobenzoxy-DL-homolysine (0.84 gm, 2.8 mmoles) was dissolved in 1N NaOH (9 ml) in an ice-bath. To this was added benzoyl chloride (0.40 ml, 3.1 mmoles) with vigorous stirring. The reaction was stirred for two hours at room temperature. The mixture was extracted with ether (2 x 10 ml) and acidified to pH 2.0 (Congo red) with 6N HCl. The white gum was extracted into ethyl acetate (3 x 25 ml) which was washed with 1N HCl, 2% $NaHCO_3$, water, and dried (Na_2SO_4). Evaporation yielded a white gum which solidified upon trituration with ether. Yield: 1.04 gm (60%) m.p. 123-124°. Found: C, 66.5; H, 6.8; N, 6.9.
$C_{22}H_{26}N_2O_5$ requires C, 66.3; H, 6.6; N, 7.0.
I.R. max: 1550(s), 1650(s), 1690(s), 1710(s).

α-N-Benzoyl-DL-homolysine·H_2O* (282.34)

This compound was prepared by the method of Ross and Green (176), as for the corresponding lysine derivative.

α-N-Benzoyl-ζ-N-carbobenzoxy-DL-homolysine (1.04 gm, 2.6 mmoles) was dissolved in glacial acetic acid and hydrogenated in the presence of 10% palladium on charcoal (0.5 gm). When the consumption of hydrogen had ceased (24 hours) the mixture was filtered through celite and evaporated. The residue was crystallized from water and air-dried. Yield: 0.51 gm (73%), m.p. 263-264°. Other preparations gave yields from 73 to 86%.

Found: C, 58.9; H, 8.1; N, 9.8. $C_{14}H_{22}N_2O_4$ requires C, 59.6; H, 7.9; N, 9.9. Weight loss at 110° *in vacuo* (P_2O_5): 6.3%; requires 6.4%.

I.R. max: 1400(s), 1490(m), 1535(m), 1550(s), 1580(s), 1630(s).

α-N-Benzoyl-DL-homolysine Methyl Ester·HCl (314.81)

This compound was prepared using α-N-benzoyl-DL-homolysine and thionyl chloride.

α-N-Benzoyl-DL-homolysine·H_2O (0.28 gm, 1 mmole) was dried *in vacuo* (P_2O_5) to remove the water. This was added to ice-cold absolute methanol (20 ml) containing purified thionyl chloride (0.09 ml, 1.2 mmole). The solution was refluxed for 1 hour and kept overnight at room temperature. The solvent was evaporated yielding a clear oil. This was evaporated repeatedly with methanol until no odor of thionyl chloride could be detected. Yield: 0.31 gm (99%). A sample was hydrolyzed as described

previously and an aliquot run on the amino acid analyzer. Found: 43.2 μmoles; requires 40.0 μmoles DL-homolysine.
I.R. max: 1450(m), 1495(s), 1540(s), 1575(m), 1600(m), 1640(s), 1735(s).

α-N-Benzoyl-ζ-N-Carbobenzoxy-DL-homolysine Methyl Ester* (412.48)

α-N-Benzoyl-ζ-N-carbobenzoxy-DL-homolysine (1.56 gm, 3.9 mmole) was dissolved in absolute methanol and cooled in an ice-bath. A solution of diazomethane in ether w added until a faint color persisted. A few drops of glacid acetic acid were added to destroy the residual diazomethane and the solution evaporated. The resulting oil was re-evaporated several times with methanol. Yield: 1.60 gm (98%).
I.R. max: 1450(m), 1540(s), 1640(s), 1690(s), 1710(s), 1735(s).

α-N-Benzoyl-ζ-N-carbobenzoxy-DL-homolysinamide* (397.46)

This compound was prepared by the method of Bergmann and Hofmann (177) as for the corresponding lysine derivative.

α-N-Benzoyl-ζ-N-carbobenzoxy-DL-homolysine methyl ester (1.60 gm, 3.8 mmoles) was dissolved in methanol saturated with ammonia. This was kept for 24 hours at room temperature in a stoppered flask. Evaporation yielded a white solid which was recrystallized from

methanol. Yield: 1.11 gm (73%), m.p. 169-171°. Found: C, 66.3; H, 7.0; N, 10.6. $C_{22}H_{27}N_3O_4$ requires C, 66.5; H, 6.9; N, 10.6.

I.R. max: 1450(w), 1470(w), 1500(w), 1520(s), 1580(w), 1635(s), 1690(s).

α-N-Benzoyl-DL-homolysinamide·HCl* (298.80)

This compound was prepared by the method of Bergmann and Hofmann (177), as for the corresponding lysine derivative.

α-N-Benzoyl-ζ-N-carbobenzoxy-DL-homolysinamide (2.0 gm, 5.0 mmoles) was dissolved in a mixture of methanol (150 ml) and 1N HCl (7.0 ml). The mixture was hydrogenated in the presence of 10% palladium on charcoal (1.5 gm), and the hydrogen was replaced frequently. When the consumption of hydrogen had ceased (24 hours), the mixture was filtered through celite and evaporated, yielding a white solid. This was recrystallized from a small volume of water. Yield: 1.20 gm (80%), m.p. 224-225°. Found: C, 55.8; H, 7.6; N, 14.3; Cl, 12.1. $C_{14}H_{22}ClN_3O_2$ requires C, 56.1; H, 7.4; N, 14.0; Cl, 11.8. pK 10.51 ± 0.04 S.E.

I.R. max: 1430(w), 1465(w), 1480(w), 1520(m), 1550(m), 1605(s), 1635(vs), 1660(s), 1670(m).

4. 2,8-Diaminooctanoic Acid and Derivatives

Benzoyl Homopiperidine* (203.28)

This compound was prepared by the method of Vogel (184), as for the corresponding piperidine derivative.

Homopiperidine (45.59 gm, 0.5 mole) was added to a mixture of NaOH (26.5 gm) in water (200 ml). The mixture was warmed to 40° and benzoyl chloride (58 ml, 0.5 mole) was added dropwise over 1 hour with vigorous stirring. The reaction was allowed to cool to room temperature, and the product was extracted into ether (3 x 100 ml). After drying (K_2CO_3) and filtering, the solvent was removed yielding a golden oil. This was distilled *in vacuo* and the product collected. Yield: 91.8 gm (90%), b.p. 150°/1.5 mm Hg, m.p. below 25°.

I.R. max: 1430(s), 1450(s), 1470(m), 1500(w), 1575(w), 1630(s).

6-Benzamido-1-chlorohexane* (239.75)

This compound was prepared by the method of von Braun (185), as for the corresponding pentane derivative.

Benzoyl homopiperidine (50.82 gm, 0.25 mole) and phosphorus pentachloride (52.07 gm, 0.25 mole) were mixed together in a round-bottomed flask fitted with a reflux condenser. The mixture was heated gently with a bunsen burner until the vigorous reaction was over. The solution

was refluxed gently for an additional 15 minutes, then cooled in an ice-bath. Ice-water was carefully added until no further reaction occurred. The oil produced was treated with steam to remove excess HCl and the mixture was evaporated. The residue was distilled *in vacuo* with great difficulty and the product was collected.

Yield: 30.0 gm (50%), b.p. >250°/2 mm Hg.

I.R. max: 1450(m), 1490(w), 1550(s), 1570(w), 1620(vs), 1700(s).

2,8-Diaminooctanoic Acid·HCl (210.71)

This compound was prepared by the method of Wada (186), as for 2,7-diaminoheptanoic acid, except that the product was isolated using Dowex 50.

To dry ethanol (75 ml) was added metallic sodium (1.73 gm, 75 mmole) and diethyl acetamidomalonate (16.3 gm, 75 mmole) followed by 6-benzamido-1-chlorohexane (23.97 gm, 100 mmole). A white precipitate appeared immediately. The mixture was refluxed for 24 hours and the solvents removed. Water (50 ml) was added to the residue and the solution was extracted with ether (4 x 75 ml), dried (K_2CO_3), and evaporated. The residual oil was boiled for 2 hours with 20% potassium hydroxide (50 ml), cooled and extracted with ether (2 x 50 ml). The aqueous phase was acidified to pH 2.0 (Congo red) with 6N HCl in an ice-bath. The gum which precipitated was

extracted into ethyl acetate (3 x 75 ml). The organic layer was washed with 1N HCl, 2% $NaHCO_3$, and water. This was dried (Na_2SO_4), filtered and evaporated, yielding a yellow oil which was refluxed overnight with 6N HCl (75 ml) and evaporated. The residue was dissolved in water (150 ml) and stirred for three hours with Dowex 50 (100 ml, 20-50 mesh, H^+ form. After stirring, the resin was filtered off, washed with water until neutral and suspended in 4N NH_4OH (150 ml). After 1 hour the resin was filtered off and the solution evaporated, yielding an oil. This was dissolved in a little water (25 ml) and the pH adjusted to 5.8 with 2N HCl. Evaporation yielded a white solid which was crystallized from water-ethanol. Yield: 0.62 gm (4%), m.p. 263-265° (lit. (188) 261-262°).
I.R. max: 1420(s), 1510(m), 1520(m), 1590(s), 1630(s).

DL-2,8-Diaminooctanoic Acid Ethyl Ester·2HCl* (275.22)

This compound was prepared by the method of Curtius and Goebel (189), using dry HCl gas and the amino acid in ethanol.

DL-2,8-Diaminooctanoic acid (0.53 gm, 2.5 mmole) was suspended in dry ethanol (20 ml) and a stream of dry HCl was passed through the mixture until a clear solution was obtained. This was kept at room temperature for 24 hours and evaporated, yielding an oil. This was re-evaporated with ethanol until no odor of HCl could be

detected. The residue was crystallized from ethanol-ether. The compound was hygroscopic and picked up one molecule of water. Yield: 0.30 gm (44%), m.p. 142-143°.
Found: C, 42.8; H, 9.1; N, 9.9. $C_{10}H_{24}Cl_2N_2O_2 \cdot H_2O$ requires C, 42.3; H, 8.9; N, 9.9.
I.R. max: 1450(w), 1470(w), 1500(s), 1600(s), 1735(vs).

5. Poly-lysines

ε-N-Carbobenzoxy-L-lysine N-Carboxy Anhydride (306.31)

This compound was prepared as described by Bergmann, Zervas and Ross (175), using α-N,ε-N-dicarbobenzoxy-L-lysine and phosphorus pentachloride. Yield: 73%, m.p. 100° (lit. (175) 100-101°).
I.R. max: 1460(m), 1535(s), 1685(s), 1775(s), 1800(s), 1850(s).

Poly-ε-N-carbobenzoxy-L-lysine

This polymer was prepared as described by Katchalski (190), using ε-N-carbobenzoxy-L-lysine N-carboxy anhydride and diethylamine as initiator. Yield: 87%.

Poly-L-lysine·HBr

This polymer was prepared as described by Katchalski (190), by treating poly-ε-N-carbobenzoxy-L-lysine with 30% HBr in acetic acid. Yield: 89%.
I.R. max: 1470(s), 1550(s), 1650(s).

ε-N-Methyl,ε-N-carbobenzoxy-L-lysine N-Carboxy Anhydride* (320.34)

This compound was prepared by the method of Fasman et al. (191), as for the corresponding ε-N-carbobenzoxy-L-lysine derivative.

ε-N-Methyl,ε-N-carbobenzoxy-L-lysine (2.40 gm, 8.1 mmole) was suspended in purified ethyl acetate (60 ml) and a stream of phosgene, first passed through concentrated sulfuric acid, was bubbled through the mixture. The ethyl acetate was kept stirring and refluxing during the addition of the gas. When all the solid had dissolved, a stream of dry nitrogen was bubbled through the solution at 40° until no phosgene remained. Petroleum ether (150 ml) was added and the mixture was cooled for several hours. The crystals were filtered off and recrystallized from ethyl acetate-petroleum ether until no chloride ion remained in the product (5 times). Yield: 2.23 gm (85%), m.p. 83°.

I.R. max: 1420(w), 1460(w), 1500(w), 1665(s), 1780(s), 1840(m).

Poly-ε-N-methyl,ε-N-carbobenzoxy-L-lysine*

This compound was prepared by the method of Katchalski (190), as for the corresponding ε-N-carbobenzoxy-L-lysine derivative.

ε-N-Methyl,ε-N-carbobenzoxy-L-lysine N-carboxy anhydride (12.23 gm, 7.2 mmole) was dissolved in purified dimethylformamide (20 ml), and a solution of diethylamine (102 mg) in dimethylformamide (1 ml) was added. The solution was kept for 48 hours at room temperature, and water (40 ml) containing concentrated HCl (0.14 ml) was added. The product which precipitated as an amorphous white solid was kept overnight and filtered, with great difficulty. Yield: 1.40 gm (69%).

Poly-ε-N-methyl-L-lysine·HBr*

This compound was prepared by the method of Katchalski (190), as for the lysine derivative.

Poly-ε-N-methyl,ε-N-carbobenzoxy-L-lysine (1.40 gm) was treated with 30% HBr in glacial acetic acid (20 ml). After the solution had stood ½ hour at room temperature, ether (150 ml) was added. The resulting oil was collected and crystallized from water-acetone. The product was hygroscopic. Yield: 1.06 gm (86%). Found: C, 34.3; H, 7.2; N, 11.6; Br, 33.0. $(C_7H_{15}Br\ N_2O\cdot H_2O)_n$ requires C, 34.9; H, 7.1; N, 11.6; Br, 33.1. M.W.~2240. I.R. max: 1470(s), 1550(s), 1650(s).

IV. Attempted Resolution of DL-Homolysine

For the purpose of kinetic studies, it was hoped that optically active derivatives could be used. Several

attempts were made to resolve DL-homolysine by conventional methods. These experiments resulted in limited success, as it was only possible to obtain a relatively pure D-isomer. The L-isomer was always highly contaminated with D-isomer.

1. Renal Acylase I

Renal acylase I has been successfully used to resolve DL-lysine by the stereospecific hydrolysis of suitable derivatives (192). The corresponding homolysine derivatives were synthesized and subjected to the enzyme.

α-N-,ζ-N-Dichloroacetyl-DL-homolysine (1.73 gm, 5.5 mmoles) was dissolved in water (40 ml) with the aid of 2N LiOH. When all the amino acid had dissolved, the pH of the solution was adjusted to 7.1 with dilute HCl. Acylase powder (Nutritional Biochemicals, Cleveland, Ohio; 14 mg) was added, and the solution incubated at 37°C. Aliquots were removed at 0, 24, 42 and 113 hours and analyzed for ζ-N-chloroacetyl-L-homolysine produced on the amino acid analyzer. Ideally the production of this compound should have continued until one half the amount of starting material had been consumed. Unfortunately the reaction rate leveled off far below this mark. This is shown in Figure 1. We have no explanation for these results.

A similar experiment using α-N-chloroacetyl-ζ-N-carbobenzoxy-DL-homolysine gave virtually identical results.

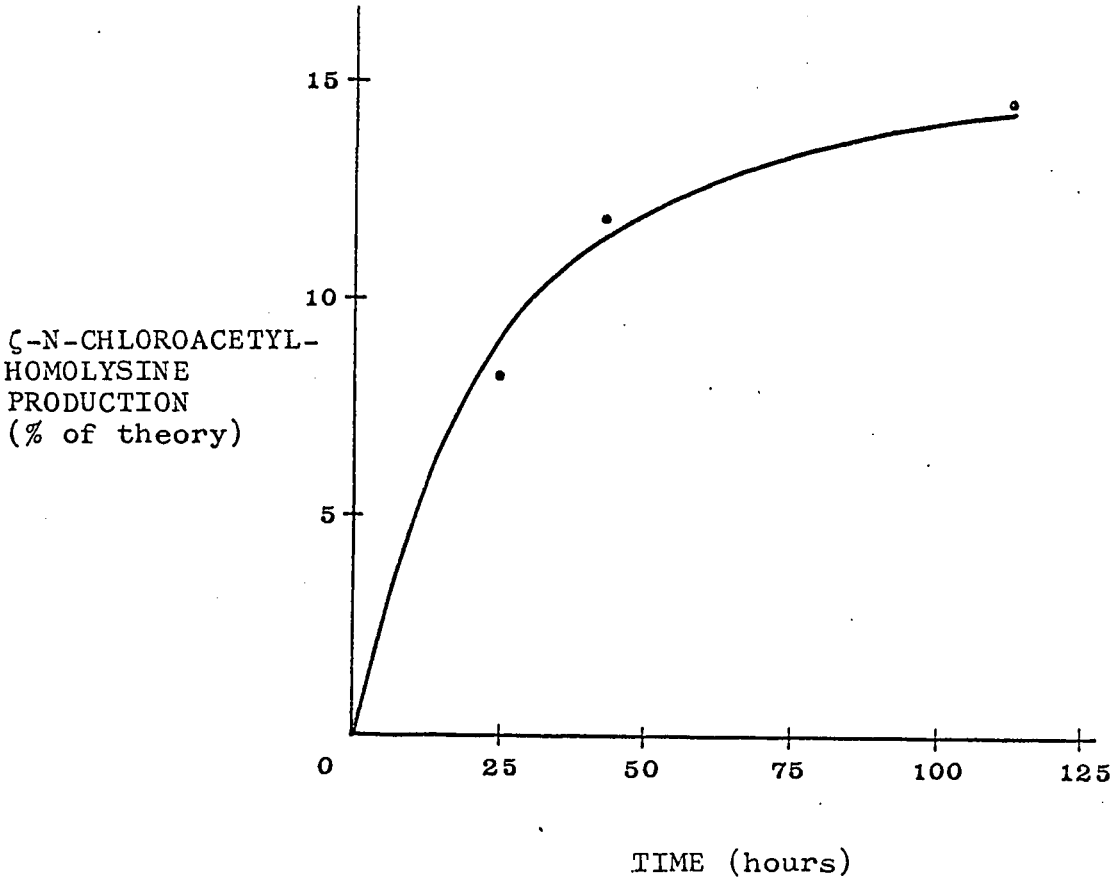


FIGURE 1 Hydrolysis of α-N,ζ-N-dichloroacetyl-DL-homolysine by renal acylase 1 at pH 7.1 and 37°.

2. Trypsin

During the course of this work pilot studies indicated that DL-homolysine ethyl ester was hydrolyzed by trypsin at an appreciable rate. This suggested a possible route to the resolution by a procedure analogous to that described by Erlanger et al. (29) for benzoyl-DL-arginine-p-nitroanilide. This involved incubating the DL-homolysine ethyl ester with trypsin and separating the L-amino acid from the D ester. The separation was accomplished on a column of Dowex 50 resin. This had proved to be an efficient method for the resolution of DL-tyrosine ethyl ester by chymotrypsin (193). The method of course depends upon the assumption that the D-isomer would not be hydrolyzed.

DL-Homolysine ethyl ester (1.0448 gm, 4.0 mmoles) was dissolved in water (4.0 ml) containing calcium chloride (25 μmoles). The pH of the solution was adjusted to 5.0 with NaOH and trypsin (Nutritional Biochemicals, Cleveland, Ohio; 2 x crystallized; 10 mg) was added. The pH was maintained with a pH-stat (Radiometer, Copenhagen) charged with 1.00N NaOH. The reaction was allowed to proceed for two hours when 1.958 ml of NaOH had been consumed (50% hydrolysis requires 2.00 ml). The solution was diluted to 25 ml with 0.2N sodium citrate buffer pH 2.2. An aliquot of this solution was run on the amino acid analyzer. This showed 2300 μmoles of "L" homolysine to be

present suggesting that some of the D ester had been hydrolyzed. The ester used initially was virtually free of contaminating amino acid.

The diluted solution was poured onto a column of Dowex 50 (15 ml, 200-400 mesh, H^+form) and rinsed with several bed volumes of pH 2.2 buffer. Citrate buffer (0.35N, pH 5.30) was passed through the column to elute the free amino acid. The progress of the elution was followed by analyzing aliquots with ninhydrin. When the effluent was ninhydrin-negative, the column was treated with 1N NaOH to remove the ester. An aliquot of the pooled free amino acid fractions indicated the presence of 2040 μmoles of "L" homolysine. The elution with NaOH was continued until no ninhydrin-positive material remained on the column. The aliquots were neutralized before the ninhydrin assay. The alkaline fraction was found to contain 1940 μmoles of "D" homolysine.

Both fractions were desalted using Dowex 50 (75 ml, 200-400 mesh, H^+ form). The solution of either the "L" or "D" amino acid was poured onto the column and rinsed in with distilled water. The column was carefully washed with distilled water until the effluent was neutral to litmus. The amino acid was then eluted off the column using 4N ammonium hydroxide. The progress of the elution was followed by evaporating aliquots on cellulose plates

and spraying with ninhydrin. When all the amino acid had been eluted, the ammonia was evaporated, yielding a white powder. This was dissolved in water, adjusted to pH 5.9 and re-evaporated. The residue was recrystallized from water-ethanol. Yield: 0.40 gm "L" homolysine (99%), 0.28 gm "D" homolysine (80%).

Conventional methods for the investigation of optical purity by polarimetry or by enzyme assays were not sensitive enough. The optical purity of these compounds was examined by the method of Manning and Moore (194). This method uses the separation of diastereoisomers formed by reacting the amino acid with an N-carboxy anhydride. When the work was initiated the details of the Manning and Moore technique were not available. As a result the following method was developed; this procedure is somewhat more laborious than that described by Manning and Moore for L-lysine, but gives similar results.

About 40 mg of the amino acid to be assayed was dissolved in boiling water, and excess basic copper carbonate was added. The mixture was allowed to cool and then filtered. The solution of the copper salt was treated with sodium bicarbonate and p-nitrophenyl acetate, exactly as described by Leclerc and Benoiton (183). The mixture was clamped in a wrist-action shaker and agitated overnight. The next day the precipitated copper salt was filtered off

and washed with ethyl acetate and water. The copper was removed in the usual way by means of hydrogen sulfide. Evaporation of the solution gave the ω-acetyl amino acid in 36 to 54% yield.

The ω-acetyl amino acid (18 mg) was dissolved in ice-cold 0.45N sodium borate buffer pH 10.5. This was added to alanine-N-carboxy anhydride (12 mg, Miles, Elkhart, Indiana) and the mixture stirred vigorously on a vortex mixer (Fisher) for several minutes. The mixture was acidified with 1 drop of concentrated HCl and diluted to 10 ml with 0.2N sodium citrate buffer pH 2.2. 0.5 ml was run on the long column of the amino acid analyzer using the pH 4.25 buffer. When the racemate was treated as described above, the two diastereoisomers separated. The data appear below:

Compound	time (min)	ratio of H x W
L-Ala-D-Lys(Ac)	49.5	--
L-Ala-L-Lys(Ac)	58.0	--
L-Ala-D-Homolys(Ac)	70.5	$\frac{1}{1.07}$
L-Ala-L-Homolys(Ac)	78.5	

Since the actual concentration of the diastereoisomers was not known, a constant could not be calculated. Instead the ratio of the height times width was determined for each peak. Using this ratio and (assuming 50% of each

isomer (racemate)), the percentage of "D"-isomer in the "L" and L in the D could be determined.

When the "L"-homolysine prepared above was analyzed, it was found to consist of 66% L- and 34% D-homolysine. The "D"-isomer was also highly contaminated. Such high contaminations can be explained in only two ways: 1) the D-isomer is hydrolyzed by trypsin or 2) the isolation procedure leads to racemization. The second possibility seemed at first to be the most likely explanation since 1) in the case of chymotrypsin the D-isomer was not hydrolyzed and 2) there was reason to suspect that treatment with resin could in some cases lead to racemization (195). This possibility was investigated by treating a sample of L-lysine exactly as the products from the resolution were treated. The lysine was eluted from both columns, isolated, and checked for optical purity by preparing a diastereoisomer as previously described. This showed that treatment with resin under these conditions does not lead to racemization. This seemed to suggest that the D-isomer had been hydrolyzed, and, as was later discovered this appears to be true.

3. L-Amino Acid Oxidase

During the course of this work it was discovered that L-homolysine was a good substrate for L-amino acid

oxidase from Crotalus adamanteus (Sigma, St. Louis, Missouri) under the conditions of Paik and Kim (196). An attempt was made to obtain pure D-homolysine both from the racemate and from partially resolved material from the tryptic digests.

"D"-Homolysine (98.34 mg, 16% L isomer) was dissolved in 0.1M tris buffer pH 8.2 (3.0 ml). A solution of purified L-amino acid oxidase (1.0 mg/1.0 ml) was added, and the solution was incubated at 37° under a stream of oxygen. After one week the mixture was evaporated and diluted with 0.2N sodium citrate buffer, pH 2.2, to 10 ml. An aliquot was run on the amino acid analyzer. Theoretically, the following reaction would be expected:

$$84\%\ \text{D-homolysine} + 16\%\ \text{L-homolysine} \xrightarrow[\text{pH } 8.2]{\text{LOX}}$$

$$84\%\ \text{D-homolysine} + 16\%\ NH_3 + 16\%\ \varepsilon\text{-aminocaproic acid.}$$

The progress of the reaction could thus be followed by measuring the production of ε-amino-n-caproic acid. In our case 18% was found and the reaction was therefore complete. When an aliquot of the reaction mixture was run on column I of the amino acid analyzer, it was found that ε-amino-n-caproic acid and D-homolysine were very

well separated. This afforded a method for the removal of the ε-amino caproic acid prior to kinetic studies on the homolysine. The separation of the two products was accomplished by concentrating the entire reaction solution to a small volume and eluting this off the amino acid analyzer columns. This of course greatly overloaded the column, but the separation was satisfactory. Fractions were collected at 10-minute intervals and those containing the D-homolysine were pooled and desalted as previously described using Dowex-50. This gave 40 mg (50%) of D-homolysine·HCl after two recrystallizations. The optical purity was checked as outlined earlier and the product was found to be free of L-isomer.

Since this procedure seemed to give reasonable results it was tried on a larger scale (1.48 gm) using the racemate. After several weeks' incubation the reaction was worked up as described above. Yield: 0.68 gm (90%). This isomer was found to contain 10% L-homolysine. The contamination probably arose from an incomplete oxidation of the L-isomer.

V. Discussion - Some Problems of Synthesis

Several problems were found with particular reactions in the synthetic section. These are now discussed one at a time:

1. Esterification of Amino Acids:

Throughout the synthetic section, both thionyl chloride and diazomethane in ether were used to esterify amino acids and derivatives. Diazomethane was used principally when an amino acid contained a labile substituent, or when the ester was required in the free base form. The esters were usually extracted from sodium carbonate into ether; in some cases, however, the scale of the experiment rendered this method of purification impractical. Furthermore, since a number of the esters used for the kinetic studies were oils, the efficiency of the esterification had to be high in order that the esters might be used without purification. The progress of esterification was investigated by following the rate of disappearance of the starting material, in a model system, using the amino acid analyzer. α-N-Benzoyl-L-lysine whose structure is typical for the amino acids we esterified, was chosen for this study even though its low solubility in alcohol renders it impossible to obtain optimal results. The reactions were carried out as follows.

To ice-cold absolute methanol was added purified thionyl chloride followed by α-N-benzoyl-L-lysine. The mixture was refluxed for 1 hour and kept at room temperature for 24 hours. Evaporation of the solvents yielded an oil, which was dissolved to 50 ml with 0.2N

sodium citrate buffer pH 2.2. An aliquot was run on column II of the amino acid analyzer and the amount of unreacted starting material measured. This experiment was repeated twice.

Similarly α-N-benzoyl-L-lysine was suspended in absolute methanol and a solution of diazomethane in ether added until all the amino acid had dissolved and a yellow color persisted. The solution was kept for $\frac{1}{2}$ hour at room temperature and evaporated. The residual oil was evaporated once more, after the addition of 1N HCl, and dissolved to 50 ml as before. An aliquot was also run on column II of the analyzer, and the amount of unreacted starting material measured. These experiments were also done in triplicate. When thionyl chloride was used, only 1.44 to 1.82% of the starting material could be found. When diazomethane was used, the residual amino acid amounted to 2.88 to 5.61%. As mentioned before, the lower yield in this case is probably due to the low solubility of the starting material. Furthermore, it was shown that the esterifications gave mainly the desired product since elution of the column with NaOH gave only one peak.

The optical rotation of the products was measured in order to see whether any gross changes of configuration had occurred. These measurements were performed in ethyl alcohol solution (C = 2) and in a 2-dm tube (Rudolph and

Sons, Model 62). In the former case the $[\alpha]_D^{22}$ was -17.0° and in the latter -16.9°, indicating no racemization.

2. Carbobenzoxylation of ε-N-Methyl-L-lysine:

When the copper salt of L-lysine is treated with carbobenzoxy chloride in the usual manner and the copper salt is subsequently removed, ε-N-carbobenzoxy-L-lysine can be secured in a 70 to 75% yield. However, treatment of the copper salt of ε-N-methyl-L-lysine under similar conditions leads to yields of ε-N-methyl,ε-N-carbobenzoxy-L-lysine in the 20-45% range. This was rather disappointing in view of the difficulty of preparing the starting material. The course of a typical reaction was followed in order to see whether the yield could be improved.

ε-N-Methyl-L-lysine·HCl (1.967 gm, 10 mmole) was dissolved in boiling water and excess basic copper carbonate was added. The mixture was boiled gently for a further ½ hour, cooled and then filtered. To this was added sodium bicarbonate (2.52 gm, 40 mmole) and carbobenzoxy chloride (1.7 ml, 12 mmole). The reaction was stirred vigorously at room temperature for three hours. At the end of this period, the precipitated copper salt was filtered off and dried in vacuo (P_2O_5). Yield: 1.20 gm. The mother liquors were assayed as follows: An aliquot was removed and freed of copper by means of hydrogen

sulfide treatment. This was filtered and run on the amino acid analyzer in the usual way. The presence of roughly equal quantities of ε-N-methyl-L-lysine and ε-N-methyl,ε-N-carbobenzoxy-L-lysine, representing slightly more than half of the initial amount of the amino acid, was thereby demonstrated.

The mother liquors were recarbobenzoxylated using sodium bicarbonate and carbobenzoxy chloride as above. After a further three hours stirring, an aliquot was removed and treated as previously described. This yielded a further 0.76 gm of the copper salt. This aliquot showed the presence of a small amount of ε-N-methyl-L-lysine and a larger amount of ε-N-methyl,ε-N-carbobenzoxy-L-lysine. The reaction was stirred overnight and worked up once more. This yielded a further 0.81 gm of the copper salt. An aliquot analyzed in the usual way contained neither ε-N-methyl-L-lysine nor ε-N-methyl,ε-N-carbobenzoxy-L-lysine. Total yield of the copper salt of ε-N-methyl, ε-N-carbobenzoxy-L-lysine: 2.87 gm (71%). The copper was removed using hydrogen sulfide and a mixture of water and acetic acid. Yield: 1.34 gm (41%).

The above experiment showed that the low yields are not due to the failure of the carbobenzoxy chloride to react, which was as expected, since it is known that sarcosine is readily carbobenzoxylated (197). The reason

for the poor yields lies in the fact that the copper salt of ε-N-methyl,ε-N-carbobenzoxy-L-lysine is more soluble in water than the corresponding non-alkylated lysine. The reaction occasionally gave higher yields when the stirring at room temperature was prolonged. It may also be noted that the carbobenzoxylation of ornithine and homolysine, like that of ε-N-methyl-L-lysine, gave yields in the 30 to 50% range (198, 199).

After several recrystallizations of the product the m.p. found was 231-233°. Benoiton and Deneault (26) report 210-212°. Both samples have identical infrared spectra but there was not enough of the original (Benoiton and Deneault) material for a melting point redetermination. We have no explanation for this difference.

3. Preparation of DL-2,8-Diaminooctanoic Acid:

This compound was not prepared by the method of Takagi and Hayashi (188) since it required the use of hydrazoic acid which is very explosive. We therefore decided to use a modification of the method of Wada (186), used to prepare DL-homolysine. In this case 6-benzamido-1-chlorohexane was used in place of 5-benzamido-1-chloropentane. The DL-2,8-diaminooctanoic acid however was only obtained in 4% yield. This was due to the fact that a) the chloro compound used for the condensation was not pure, and b) one of the intermediates was erroneously

washed with 2% sodium bicarbonate. Nevertheless we secured enough material for our purposes.

Enzymatic Studies

I. The Action of Some Enzymes on Homolysine Derivatives:

In addition to the enzymes already mentioned in the discussion of the attempted resolution of homolysine, the activities of several other lysine-directed enzymes were tested using appropriate homolysine derivatives.

a) L-Amino Acid Oxidase

As previously mentioned, homolysine was a substrate for L-amino acid oxidase from Crotalus adamanteus. This activity was measured as described by Paik and Kim (196) using a Warburg respirometer.

The amino acid was dissolved in distilled water at a concentration of 50 μmoles per milliliter (based on the L-isomer). This solution (1.0 ml) was added to 0.2M glycine buffer pH 9.0 (1.0 ml) in a Warburg flask. Twenty percent potassium hydroxide was added to the center well to absorb any carbon dioxide present. The enzyme solution (0.5 mg/ml, 1.0 ml) was added and the mixture incubated at 37°. The rate of oxygen uptake was measured every 2.5 minutes for 10 minutes. A blank containing no substrate and a control with L-lysine were run in parallel. All samples were run in duplicate. The volume of oxygen consumed was calculated as described in Manometric Techniques (200) using a flask constant. The rates are expressed in micromoles of oxygen consumed per hour per milligram enzyme used. This gave a rate of 79.8 for homolysine and 70.7 per lysine. The value

given by Paik and Kim (196) for lysine is 99.0.

b) L-Lysine Decarboxylase

The activity of lysine decarboxylase towards DL-homolysine was tested as described in Chemistry of the Amino Acids (201).

A crude acetone powder of lysine decarboxylase (100 mg, Sigma Chemical Co.) was stirred for two hours at 27° with 0.03M phosphate buffer, pH 8.0 (5.0 ml). This preparation was centrifuged (10,000 r.p.m.) and the clear supernatant fraction used. The activity was measured by incubating in a Warburg respirometer at 37°. A 0.06M substrate solution (0.5 ml) was mixed with the enzyme preparation (0.5 ml) in 0.2M phosphate buffer pH 6.0 (2.0 ml). A blank containing no substrate and a sample containing L-lysine were run in parallel. The enzyme decarboxylated lysine but had no effect on homolysine. Ornithine is also resistant to this enzyme as are all N-substituted derivatives of lysine (202).

c) ε-Lysine Acylase from Hog and Chicken

The activity of ε-lysine acylase from hog and chicken towards ζ-N-acetyl-DL-homolysine was determined by the method of Leclerc and Benoiton (203). Dr. Jean Leclerc kindly donated samples of the partially purified enzymes.

The hog enzyme (100 mg) was dissolved in 0.1M glycine buffer, pH 8.0 (1.0 ml) and added to a solution of the substrate at 37°. The reaction was stopped three hours later by adding a solution of 20% sulfosalicylic acid (1.0 ml). The mixture was cooled in a refrigerator for ½ hour to ensure complete precipitation of the protein, and filtered through celite. A blank containing water in place of the substrate, and an assay with ε-N-acetyl-L-lysine, were run in parallel. The activity was measured by determining the amount of free amino acid produced by the enzyme, using the amino acid analyzer. The enzyme was active upon the lysine derivative but inactive upon the homolysine derivative. δ-N-Acetyl-L-ornithine is also resistant to the enzyme (204).

The chicken enzyme was assayed in a similar manner. The enzyme solution (4.85 mg/ml) in 0.1M glycine buffer pH 9.0 (1.0 ml) was added to the substrate solution and assayed under the conditions described above. In this case both compounds were hydrolyzed by the enzyme. The homolysine derivative was hydrolyzed at a rate exactly one half of that for lysine. δ-N-Acetyl-L-ornithine and γ-N-acetyl-L-diaminobutyric acid are hydrolyzed at about the same rate as lysine (203).

II. Trypsin

1. Action on Amino Acid Esters

a) Materials and Methods:

Trypsin was purchased as the three times crystallized form, lyophilized in lots of 50 gm (Worthington, lot 7JA). The enzyme was weighed, dissolved in 0.001N HCl, (pH 3.0) and the concentration was determined by measuring the optical density at 280 mμ and using 0.694 as the factor for conversion to mg/ml (68). The molecular weight of the enzyme was assumed to be 23,800 (205). The optical densities were measured on a Zeiss model PMQ II spectrophotometer. The enzyme solution was used within six hours of preparation.

Most of the kinetic experiments were performed in the presence of 0.1M sodium chloride and 5mM calcium chloride. A stock solution of these two components (1.0M in NaCl and 0.05M $CaCl_2$) was added to the reaction mixtures by means of an automatic pipette (Zipette, Canlab). The accuracy of the automatic pipette was verified by pipetting water into preweighed vials and reweighing. The reproducibility and accuracy compared favorably with those of an ordinary pipette.

Since the hydrolysis of esters by trypsin leads to the production of protons, the rate of the reaction could

be conveniently measured using a pH-stat. This was done essentially according to the method of Linderstrom-Lang et al. (206). In some cases the titration of the protons produced was not quantitative due to buffering by the substrate. This depended upon the ionization constants of the reactants and products, and was only significant in the case of free amino acid esters. The hydrolysis of an ester in neutral solution may be written as:

$$R_1COOR_2 + H_2O \longrightarrow R_1COO^- + H^+ + R_2OH$$

Once the reaction was initiated by the addition of the enzyme, the pH was maintained at the desired value by continuous addition of alkali. The amount of alkali consumed per unit time was monitored by a recorder.

A photograph of the apparatus used appears in plate I. The entire unit was manufactured by Radiometer (Copenhagen) and consisted of the following:

(1) pH Meter (Model 28b). The pH meter had a reproducibility of ± 0.05 pH unit. The meter was equipped with calomel (Radiometer, K 4112) and glass (Radiometer, G2222B) electrodes. The instrument was standardized before and after each run using a standard buffer (Beckman, pH 7.0 at 25°). If the reading had changed by more than 0.05 pH unit during the run, the results were discarded. The meter was allowed to warm up overnight before use.

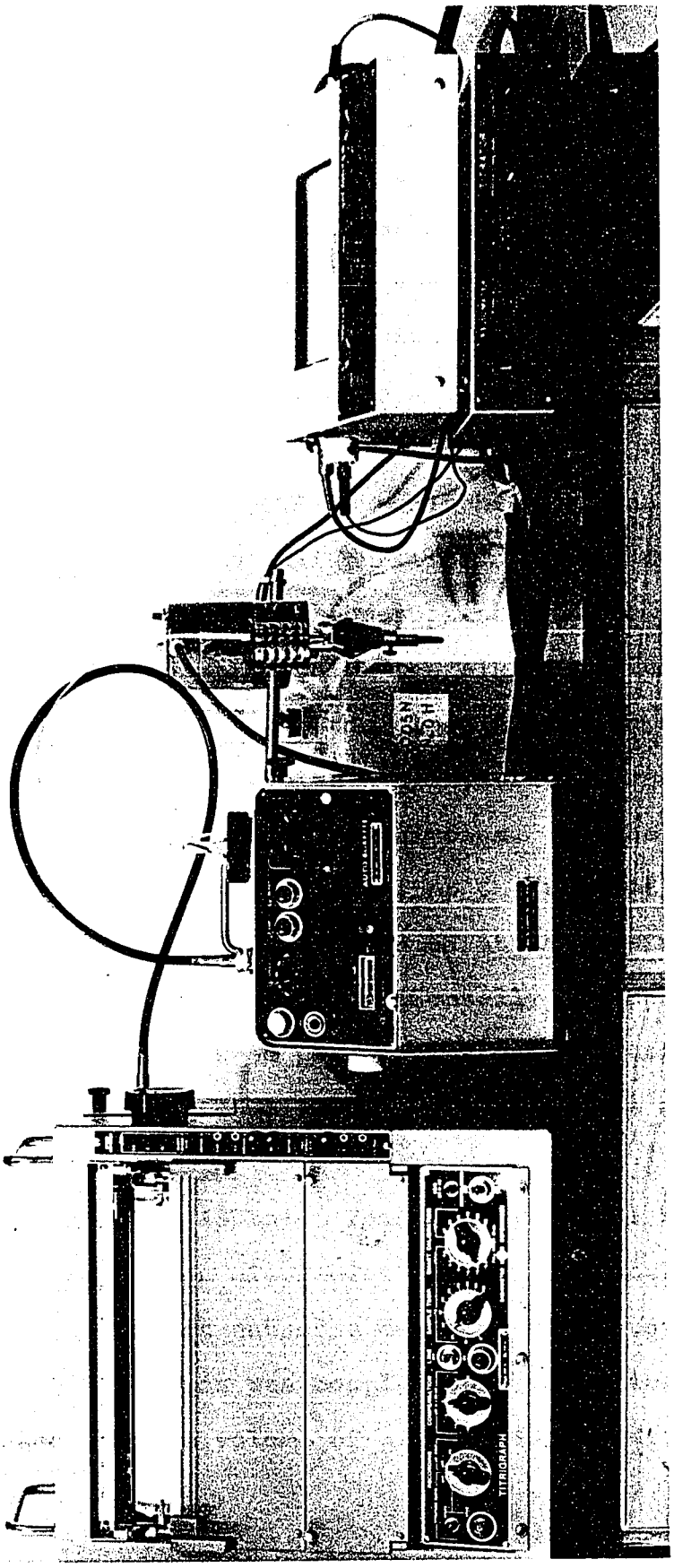


Plate 1

(2) Titrator (Model TTT 11a). This transistorized unit contained the controls for setting both the end point and the rate of alkali addition to one side of the desired pH. The endpoint desired was set directly onto the pH meter. This setting was such that after a drop of 0.05 pH unit, a relay would trigger a valve which allowed the addition of alkali to the reaction. For this reason the end point was set 0.05 pH unit higher than the desired value. The rate of alkali addition near the desired pH was adjusted by means of a proportional band. This controlled the point at which the alkali addition became maximal. If this was set too close to the pH of the end point the reaction was always overtitrated, resulting in a recording resembling a staircase. If the control was set too far from the end point the rate of alkali addition failed to maintain the pH at the desired value. The control was adjusted experimentally in such a way as to maintain the desired pH. The entire titration was controlled from this unit which contained the master start button.

(3) Auto burette (ABU1a). This unit was made up of a motor-driven piston displacing alkali in a sealed chamber and thus forcing it out of a delivery tip into the reaction mixture. The piston was coupled to a mechanical counter which recorded the volume of alkali dispensed. The

operation of the piston motor was controlled by a signal from the titrator unit. The rate of alkali addition could also be regulated by a speed setting on the unit. This was adjustable from 10 to 160% of the burette capacity per minute. This too was adjusted so that the rate of alkali addition was sufficient to maintain the pH.

The burette used throughout the kinetic experiments had a capacity of 0.25 ml. The amount of alkali added depended on the substrate concentration used in the reaction mixture. In order to get an appreciable addition, the sodium hydroxide used had to be fairly dilute. Two concentrations were used, depending on the value of Km for the reaction. For substrates with a low Km (10^{-4} to 10^{-6} moles l^{-1}) 0.005N NaOH was used, and for substrates with higher Km's (10^{-2} to 10^{-3} moles l^{-1}) 0.05N NaOH was used. The sodium hydroxide was prepared from freshly boiled distilled water and stored in polyethylene bottles protected by soda-lime guard tubes.

(4) Recorder (Model SBR2). The recorder pen drive unit was coupled directly to the burette piston motor via a flexible shaft. The chart speed was varied from 1 to 4 cm per minute depending on the rate of the reaction. All reactions were allowed to proceed until the pen had traversed at least 15% of the chart paper.

(5) Titration vessel (Model TTA-31). This vessel had a capacity of 25 ml. The solutions were stirred magnetically from the bottom using round polyethylene-covered magnets. The cover of the vessel contained holes for the electrodes, the delivery tip, and the nitrogen inlet.

In general, the same procedure was followed for each set of runs. The esters were dissolved in distilled water (>0.75 megohm conductivity) and the sodium and calcium chlorides added. The pH of the solution was adjusted close to the desired value by using more concentrated alkali. This was done so as to minimize the change in the solution volume. The pH-stat was then turned on and the instrument was allowed to bring the solution to the exact pH desired. At this point a stream of nitrogen gas saturated with water vapor was blown over the surface of the solution. This prevented any atmospheric carbon dioxide from being absorbed. The recorder was turned on and the presence of any spontaneous hydrolysis was checked by allowing the reaction to stir for several minutes. Once it was certain that the system was sufficiently stable, alkali was added to bring the pH 0.05 unit above the end point. The enzyme, dissolved in 0.001N HCl, was then added using a micropipette. Almost immediately the reaction began and a tracing of alkali addition was made. After an appropriate movement of the

pen the instrument was stopped and the pH meter restandardized. This procedure was repeated through an entire series of substrate concentrations. An enzyme blank was also run for each concentration. Only at very high enzyme concentrations was there uptake of alkali. In those cases where there was hydrolysis the blank rate was subtracted, resulting in a change of only a few percent.

The slope of each curve was calculated by drawing a straight line through the recording trace. The velocity was calculated from these lines. This gave the rate in micromoles per minute per reaction volume. These were expressed per liter, multiplying by the appropriate factor.

The concentration of the sodium hydroxide in the burette was determined after each set of runs, by titration against potassium acid phthalate to pH 8.6 (phenolphthalein) with the pH-stat. The volume of sodium hydroxide required to titrate a water blank to this pH was subtracted from the volume required by the sample. A typical determination using four samples and four blanks is shown below.

Potassium acid phthalate (40.72 mg) was dissolved in 200 ml of distilled water and 2.0 ml was titrated to pH 8.6. Blanks consisting of 2.0 ml distilled water were

similarly treated.

blanks (ml)	phthalate (ml)
0.0220	0.4569
0.0209	0.4559
0.0224	0.4431
0.0235	0.4411
0.0220 ± 0.0009 S.E.	0.4518 ± 0.0057 S.E.

0.4298 ml NaOH was required to titrate 1.99×10^{-6} moles of potassium acid phthalate. The concentration of the alkali was $4.63 \pm 0.06 \times 10^{-3}$ M. The same standardization procedure was used for the 0.05N NaOH.

In general the range of substrate concentrations used was chosen so as to run from 0.5 to 5.0 times Km. This range was determined experimentally for each substrate.

Throughout the kinetic experiments a ratio of at least 100 to 1 was always maintained between substrate and enzyme concentrations. The enzyme was added in 20 to 200 microliter volumes.

A number of the esters synthesized were not hydrolyzed by trypsin under the conditions employed. In these cases the conditions were so chosen that both substrate and enzyme concentrations were higher than those necessary to detect the hydrolysis of even a poor substrate.

Each determination of the kinetic parameters was repeated from two to five times and the average result

was calculated. The kinetic values were calculated by a modified Eadie plot (Woolf plot). In this case S/V was plotted against S. The best straight line through the experimental points was obtained by the method of least squares, using a desk-top computer (Olivetti Underwood, Programma 101). The following formulae were used:

$$y = ax + b$$

where

$$a = \frac{n\sum xy - \sum x \sum y}{n\sum x^2 - (\sum x)^2}$$

$$b = \frac{\sum y \sum x^2 - \sum xy \sum x}{n\sum x^2 - (\sum x)^2}$$

b) Kinetics of Hydrolysis of Esters with a Free α Amino Group:

Before any kinetic work was undertaken with these esters, an investigation of the pH optimum of the reaction was carried out. This was done in view of the work of Goldenberg and Goldenberg (63) who showed that the pH optimum for the trypsin catalyzed hydrolysis of arginine methyl ester was 5.8. The pH optimum for the trypsin-catalyzed hydrolysis of N-acetyl substrates on the other hand lies between 7 and 8.5. Since most of the esters to be tested were derivatives of L-lysine the optimum was investigated using L-lysine ethyl ester at 1×10^{-2}M. The results of this experiment appear in Figure 2. The pH optimum was 6.2 at 25°.

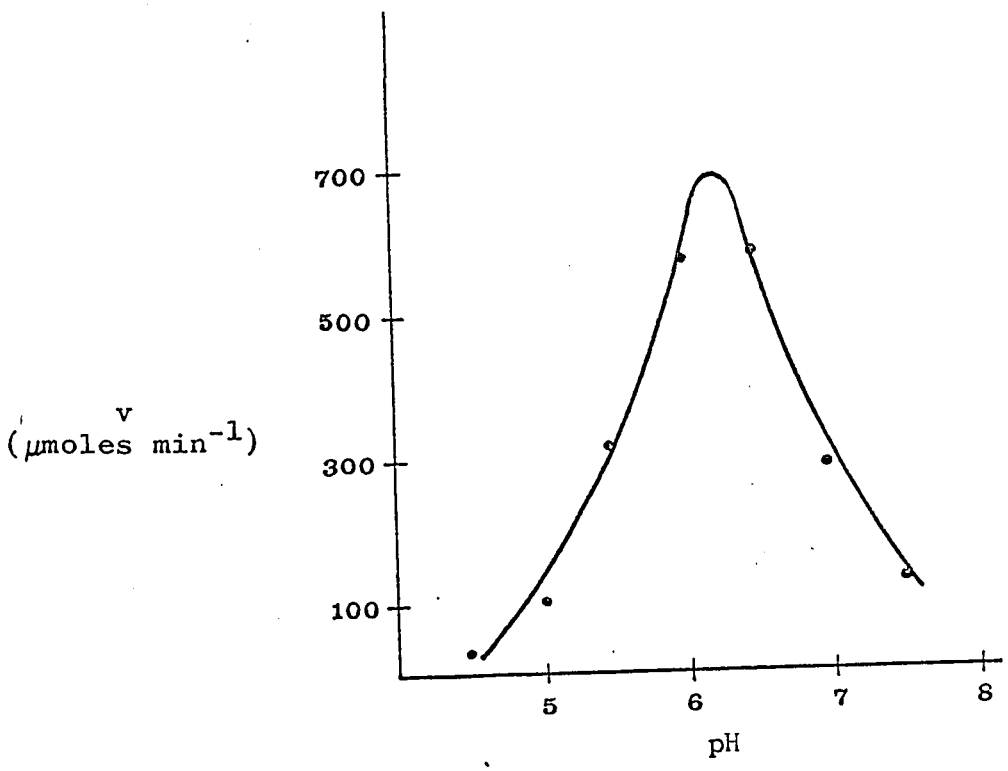


FIGURE 2 Effect of pH on the rate of hydrolysis of L-lysine ethyl ester by trypsin at 25°. [S] = 1 x 10^{-2}M; [E] = 1.0 x 10^{-6}M

The presence of free α-amino groups on these esters results in a certain amount of buffering, which causes a corresponding decrease in the release of protons observed (193). Since the pK of the α-amino group of lysine was about 8.95, this effect was negligible.

The following esters were tested as substrates, with L-lysine ethyl ester being included for comparative purposes:

L-Ornithine ethyl ester was not hydrolyzed by trypsin at a substrate concentration as high as 0.1M and an enzyme concentration of 4.71×10^{-5}M.

L-Lysine ethyl ester was a good substrate; the plotted data appear in Figure 3.

ε-N-Methyl-L-lysine ethyl ester was a substrate; the plotted data appear in Figure 4.

ε-N,ε-N-Dimethyl-L-lysine ethyl ester was not a substrate at concentrations as high as 2×10^{-2}M and an enzyme concentration of 4.71×10^{-5}M. There was an initial uptake of alkali due to the small amount of contaminating lysine ethyl ester but once this was hydrolyzed, alkali consumption ceased.

ε-N-Formyl-L-lysine methyl ester was not hydrolyzed by trypsin at substrate concentrations as high as 1×10^{-2}M and an enzyme concentration of 1.12×10^{-5}M. There was an initial uptake of alkali due to some contaminating

lysine methyl ester produced by the breakdown of the unstable ε-N-formyl compound during the synthesis. Once the lysine methyl ester was exhausted the addition of alkali ceased. The contamination, calculated from the graph, was 3.34 μmoles (6.6%), while that found on the amino acid analyzer was 3.46 μmoles (6.9%).

DL-Homolysine ethyl ester was a good substrate; the plotted data appear in Figure 5.

DL-Diaminooctanoic acid ethyl ester was not hydrolyzed by trypsin at substrate concentrations as high as 1×10^{-2}M and enzyme concentrations of 4.83×10^{-6}M.

A summary of the kinetic data appears in Table III.

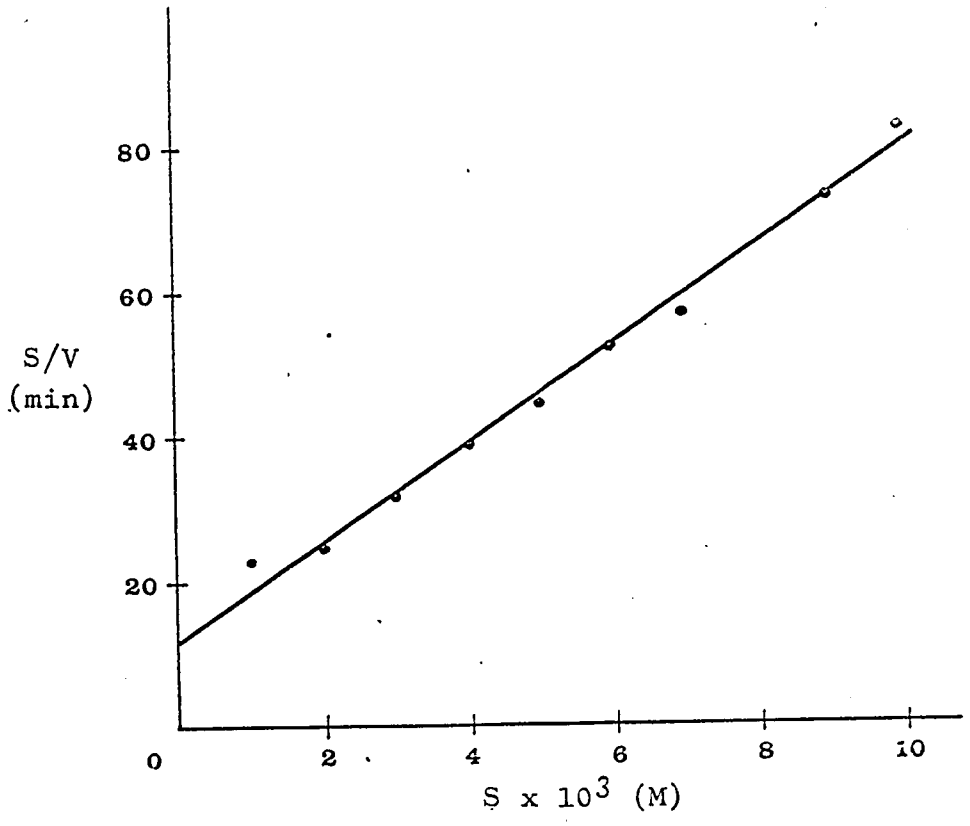


FIGURE 3 Hydrolysis of L-lysine ethyl ester by trypsin at pH 6.20 and 25°. [S] = 1 to 10 x 10^{-3}M; [E] = 1.68 x 10^{-7}M.

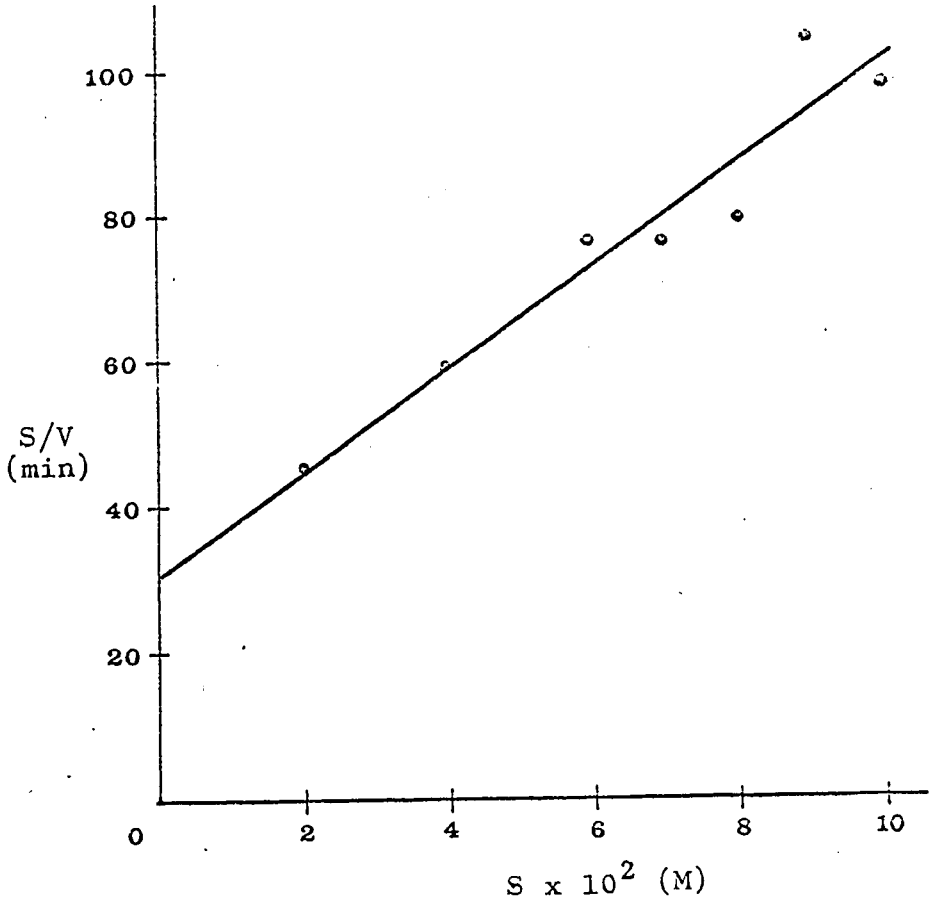


FIGURE 4 Hydrolysis of ε-N-methyl-L-lysine ethyl ester by trypsin at pH 6.20 and 25°. [S] = 2 to 10 x 10^{-2}M; [E] = 2.26 x 10^{-5}M.

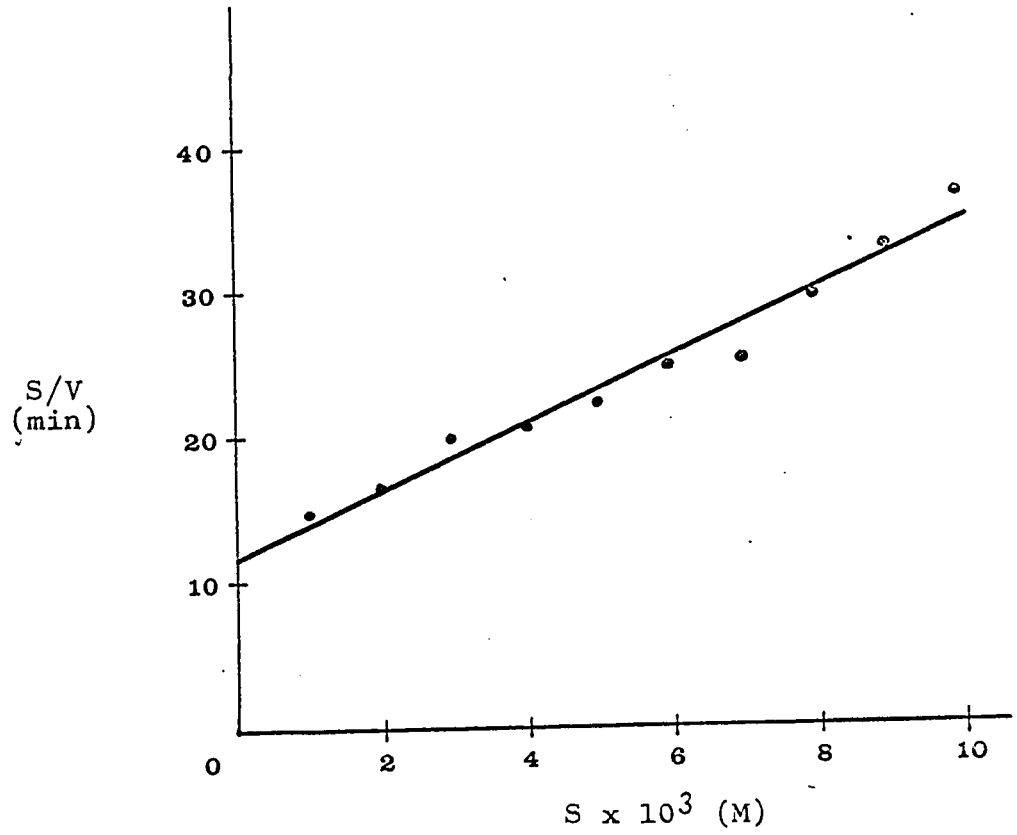


FIGURE 5 Hydrolysis of DL-homolysine ethyl ester by trypsin at pH 6.20 and 25°.
[S] based on L isomer = 1 to 10 x 10^{-3}M.
[E] = 7.47 x 10^{-6}M.

Table III

Kinetic Data for the Hydrolysis of Free Esters by Trypsin at pH 6.20 and 25°

Compound	Km(app) (moles l^{-1})	Vmax (moles min^{-1})	k_{cat} (min^{-1})
L-Ornithine Ethyl Ester	not a substrate		
L-Lysine Ethyl Ester	1.82×10^{-3}	1.48×10^{-4}	878.0
ε-N-Methyl-L-lysine Ethyl Ester	44.3×10^{-3}	14.2×10^{-4}	62.8
ε-N,ε-N-Dimethyl-L-lysine Ethyl Ester	not a substrate		
ε-N-Formyl-L-lysine Methyl Ester	not a substrate		
DL-Homolysine Ethyl Ester	5.27×10^{-3}	4.41×10^{-4}	59.0
DL-Diaminooctanoic Acid Ethyl Ester	not a substrate		

c) Kinetics of Hydrolysis of Esters with an α-N-Benzoyl Group:

The hydrolysis of these esters was measured at pH 7.0 because benzoyl-L-ornithine was included as one of the substrates. Above neutrality this substrate lactamizes (116). Furthermore, this pH was low enough to avoid any spontaneous hydrolysis of the ester.

α-N-Benzoyl-L-ornithine methyl ester was hydrolyzed slowly by trypsin. The hydrolysis only became apparent at a substrate concentration of 1×10^{-2}M and with an enzyme concentration of 2×10^{-5}M. Above this substrate concentration the compound was not soluble.

α-N-Benzoyl-L-lysine methyl ester was a good substrate. The data are plotted in Figure 6.

α-N-Benzoyl-ε-N-methyl-L-lysine methyl ester was hydrolyzed by trypsin. The results are plotted in Figure 7.

α-N-Benzoyl-ε-N,ε-N-dimethyl-L-lysine methyl ester was not hydrolyzed by trypsin at substrate concentrations as high as 1×10^{-3}M and an enzyme concentration of 3.60×10^{-6}M. Furthermore, the hydrolysis of α-N-benzoyl-L-lysine methyl ester at 1 to 8×10^{-5}M was not inhibited by the presence of 1×10^{-3}M of the dimethyl derivative.

α-N-Benzoyl-ε-N,ε-N,ε-N-trimethyl-L-lysine methyl ester was not hydrolyzed by trypsin at an enzyme concentration of 3.6×10^{-6}M and substrate concentrations of up to 1×10^{-3}M. Furthermore hydrolysis of α-N-benzoyl-L-lysine methyl ester from 1 to 8×10^{-5}M was not inhibited by the presence of 1×10^{-3}M of the trimethyl derivative.

α-N-Benzoyl-DL-homolysine methyl ester was a good substrate. The results are plotted in Figure 8.

A summary of the kinetic data appears in Table IV.

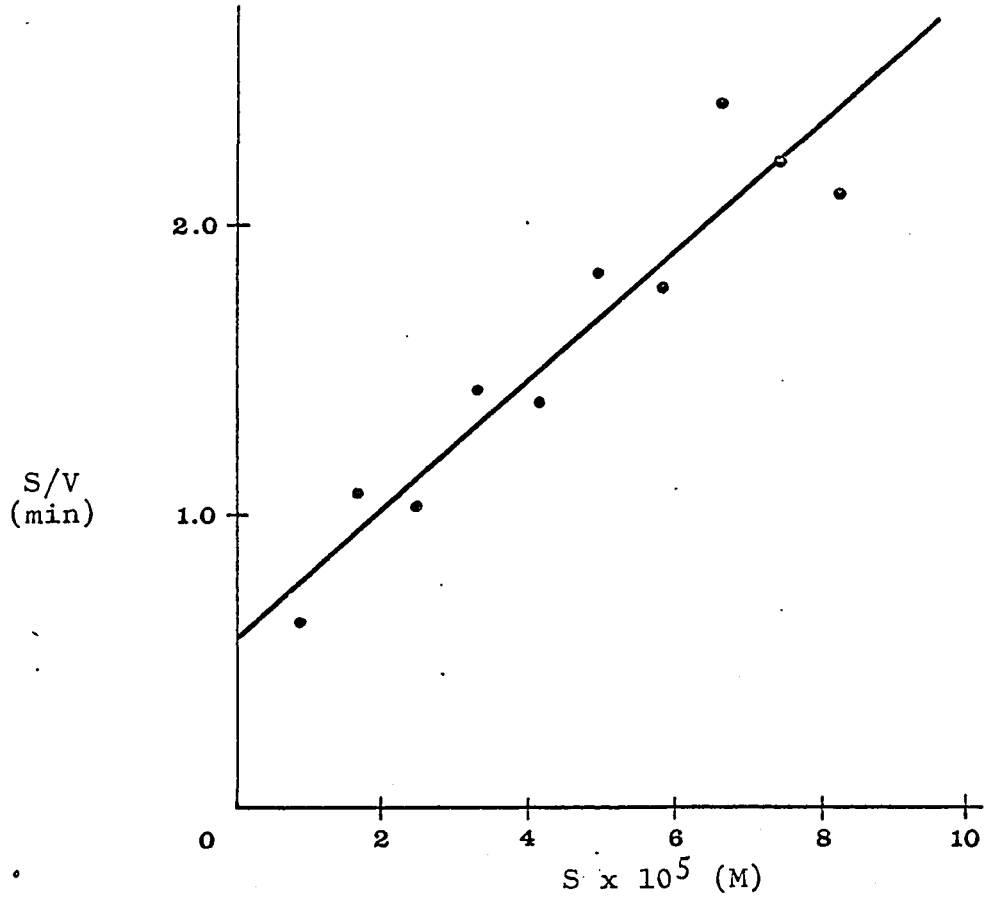


FIGURE 6 Hydrolysis of α-N-benzoyl-L-lysine methyl ester by trypsin at pH 7.00 and 25°. [S] = 1 to 10 x 10^{-5}M; [E] = 8.8 x 10^{-8}M.

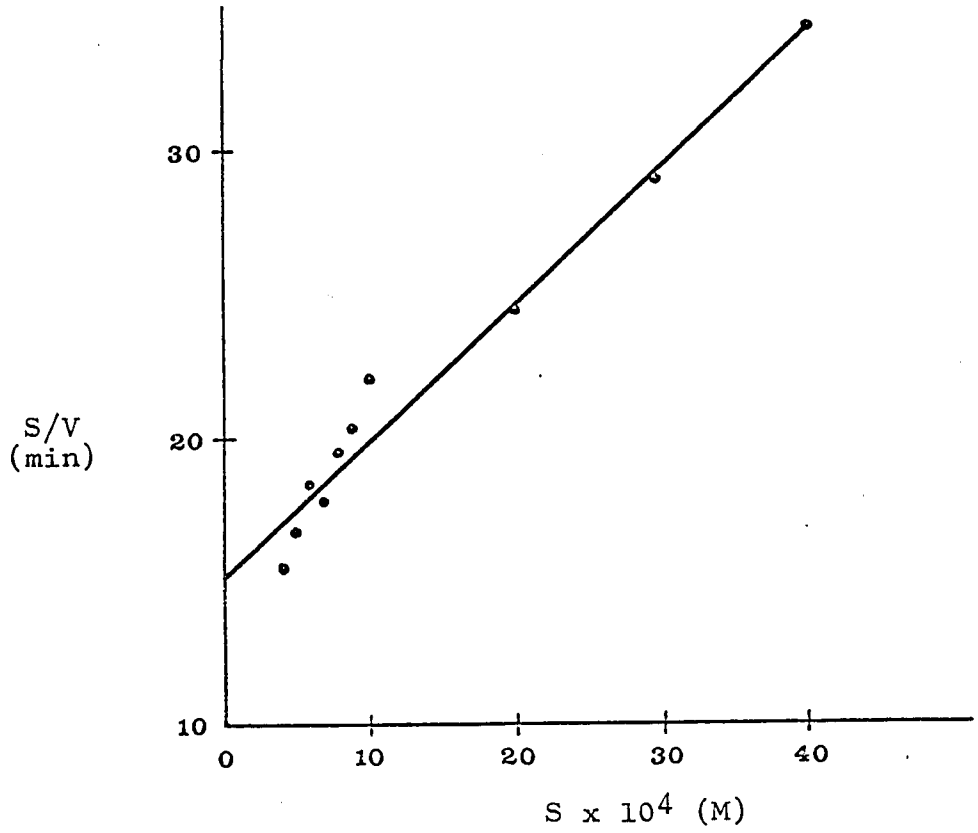


FIGURE 7 Hydrolysis of α-N-benzoyl-ε-N-methyl-L-lysine methyl ester by trypsin at pH 7.00 and 25°. [S] = 4 to 40 x 10^{-4}M. [E] = 1.11 x 10^{-6}M.

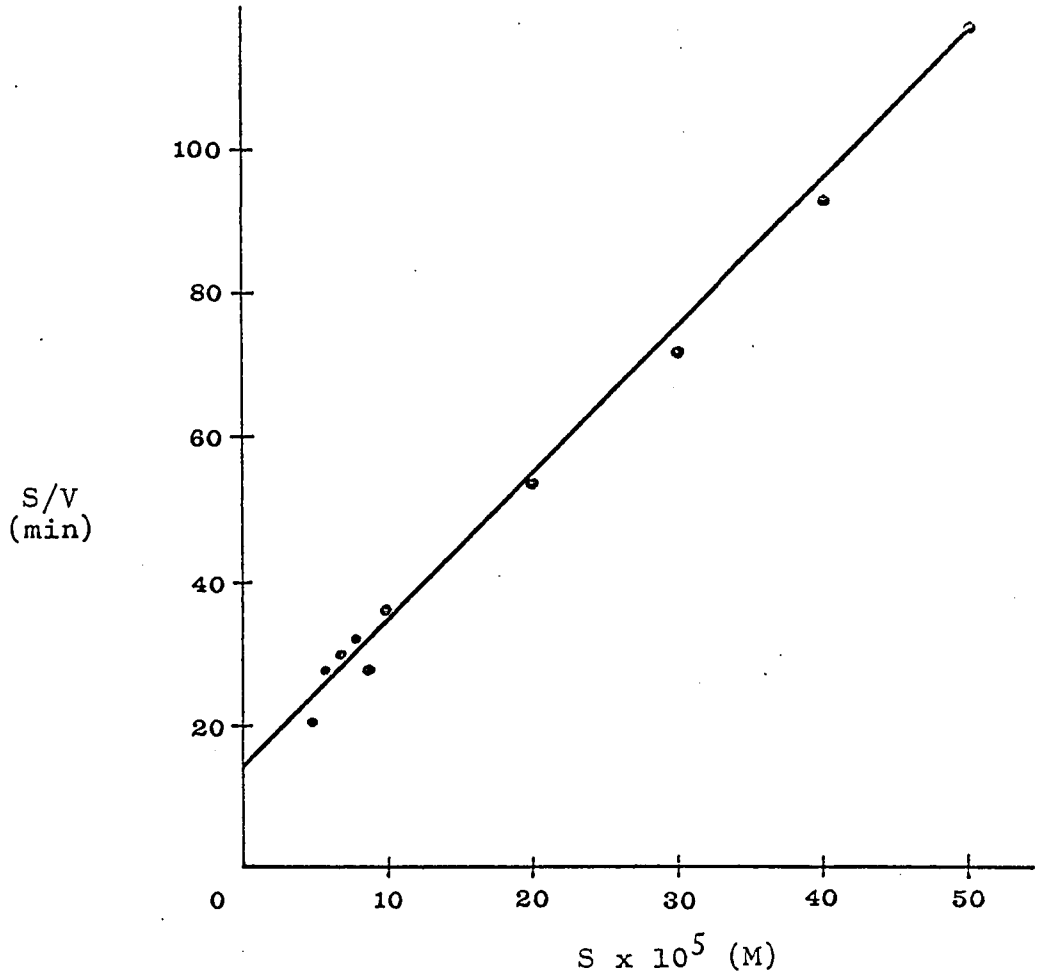


FIGURE 8 Hydrolysis of α-N-benzoyl-DL-homolysine methyl ester by trypsin at pH 7.00 and 25°. [S] based on L isomer = 5 to 50 x 10^{-5}M; [E] = 1.08 x 10^{-6}M.

Table IV

Kinetic Data for the Hydrolysis α-N-Benzoyl Esters by Trypsin at pH 7.00 and 25°

Compound	Km(app) (moles l^{-1})	Vmax (moles min^{-1})	k_{cat} (min^{-1})
α-N-Benzoyl-L-ornithine Methyl Ester	> 1 x 10^{-2}	---	--
α-N-Benzoyl-L-lysine Methyl Ester	2.78 x 10^{-5}	4.60 x 10^{-5}	519.0
α-N-Benzoyl-ε-N-methyl-L-lysine Methyl Ester	319.0 x 10^{-5}	20.8 x 10^{-5}	187.2
α-N-Benzoyl-ε-N,ε-N-dimethyl-L-lysine Methyl Ester	not a substrate		
α-N-Benzoyl-ε-N,ε-N,ε-N-trimethyl-L-lysine Methyl Ester	not a substrate		
α-N-Benzoyl-DL-homolysine Methyl Ester	6.73 x 10^{-5}	4.89 x 10^{-5}	45.0

2. Action on Amino Acid Amides

The action of trypsin on the amides was determined by measuring the amount of ammonia produced by the hydrolysis. The hydrolysis of such an amide may be written as:

$$R\text{-}CONH_2 \xrightarrow{H_2O} R\text{-}COOH + NH_3$$

There are several colorimetric methods available for determining ammonia in solution. The reagents required may be added directly to the assay mixture, or the ammonia may be separated from the mixture prior to the addition. Although the former method is more convenient because of its simplicity, it suffers from one serious drawback. The most frequently used reagents for the determination of ammonia are Nessler's reagent (207) and the phenol-hypochlorite reagent. However, it has been shown that for both of these method the presence of organic nitrogen compounds in the assay mixture interferes with the color development (208-211). It is for this reason that methods in which the ammonia is first removed have been developed. Nevertheless a number of workers have attempted to find reagents which either would destroy the interfering substances or would not be affected by them (212-214). These methods were of special interest to us since our assays would contain large amounts of this type of compound, namely the

substrate. All of these methods proved to be unsatisfactory for our purposes. A recent publication by Reardon et al. (215) suggested that an isocyanurate-salicylate reagent would be suitable for measurements in the presence of amino acids. We investigated the possibility of using this method, but found that it also suffered from the same problems as other methods. The effect of a protein (trypsin) and an amino acid (alanine) on the determination of a constant amount of ammonia appears in Figures 9 and 10.

Thus the removal of the ammonia from solution appeared to be the most satisfactory method.

The microdiffusion technique of Conway (216) and Hirahara and Seligson (217) is based upon the distillation of the ammonia from a solution. In an excellent article (218) Cedrangolo et al. pointed out the merits and drawbacks involved in the use of this method for the determination of the ammonia produced from enzymatic reactions. The main problems were (a) the necessity of rigid control of pH and temperature and (b) the time required for total recovery of the ammonia. The ideal conditions required diffusion in a rotating drum at 37° for two hours at pH 10.8. An added difficulty in the case of trypsin was the problem of stopping the enzyme reaction. This is usually done with an acid. This method

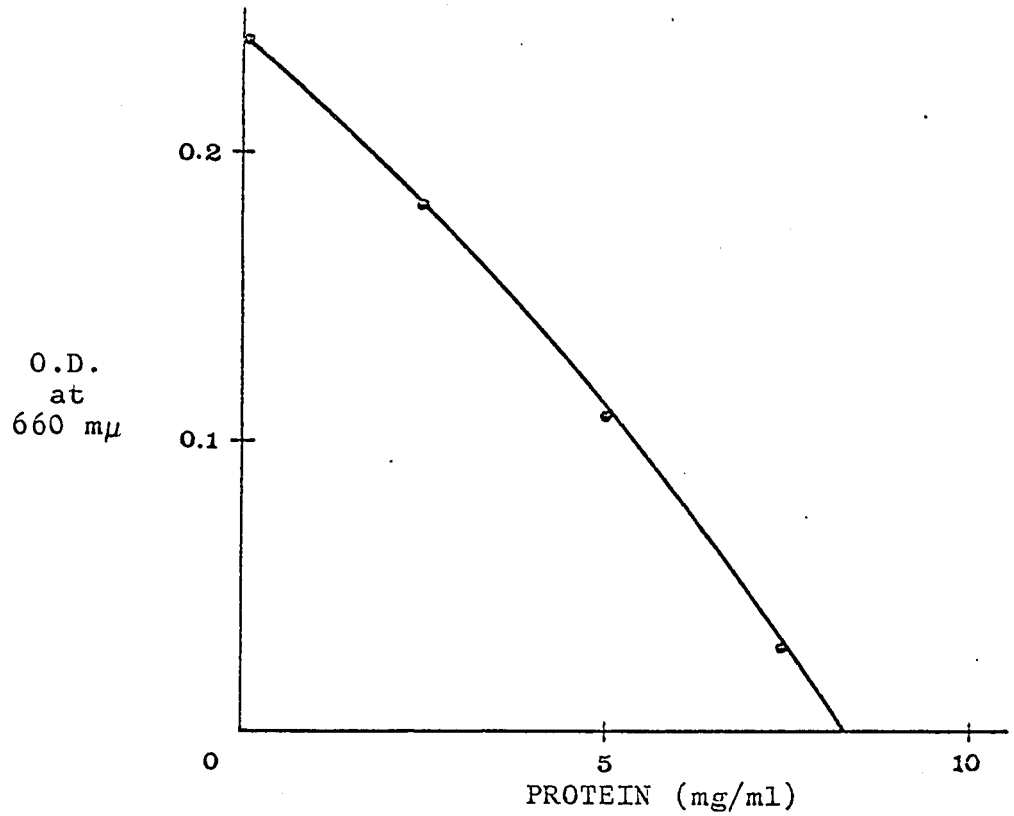


FIGURE 9 Effect of a protein (trypsin) on the analysis of 0.1 micromole of ammonia by the isocyanurate method.

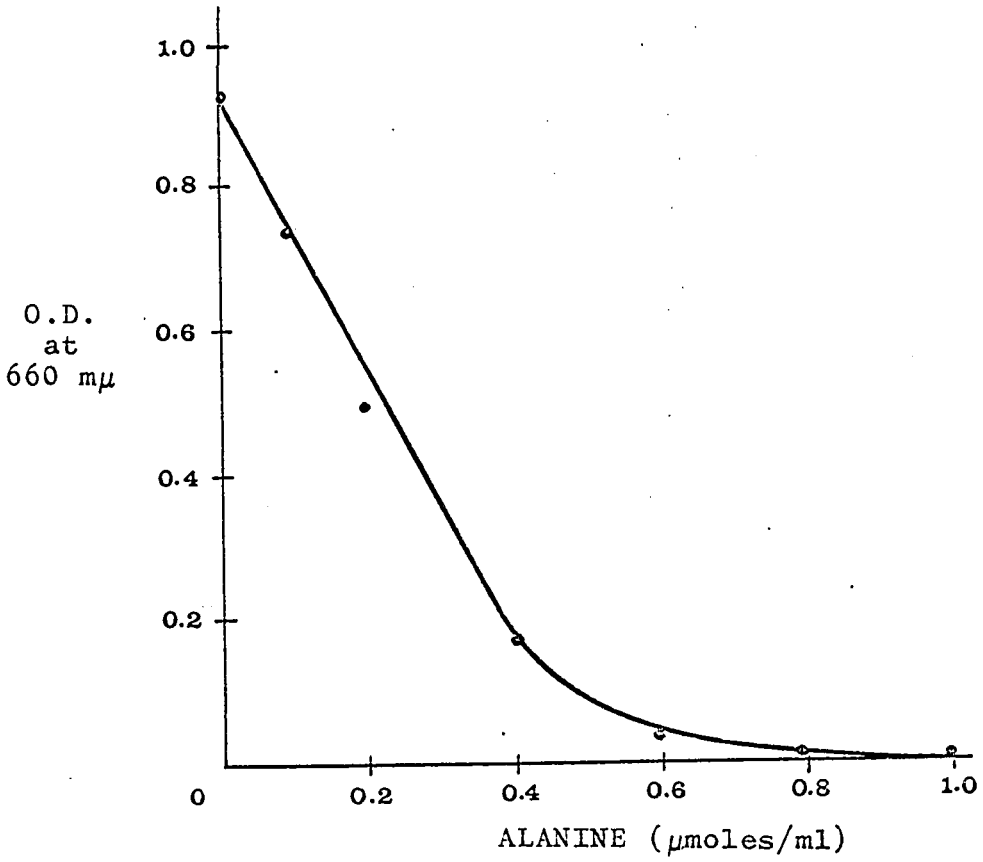


FIGURE 10 Effect of alanine on the analysis of 0.4 micromoles of ammonia by the isocyanurate method.

required that the sample be acidified, filtered, neutralized and then brought to pH 10.8. Furthermore, the reproducibility of the method is not very good, necessitating the inclusion of an internal standard. For these reasons another method was selected.

Several methods have been published using Technicon Auto Analyzers. In these cases the ammonia was removed using the dialysis units of the instrument (219,220). The shortcomings of these methods are that the interfering substances could also diffuse through the membrane. Once the ammonia was diffused out of the reaction mixture one of the normal colorimetric methods was applied. If any of the substrate molecules had passed through the membrane, there was the possibility of interference.

The last remaining method involves the isolation of the ammonia on an ion exchange resin (221). This may be done by pouring the solution to be determined onto the resin, isolating the ammonia and measuring the amount present using one of the conventional reagents. Such a batchwise process would have been tedious. The simplest and most straightforward method made use of an amino acid analyzer column (222). Whitaker and Jurasek applied five samples to the long column of a Beckman Model 120B at twenty minute intervals. The five peaks were eluted after some five hours. Apart from being very time consuming,

the method appeared to be free of the problems found with other determinations.

The method we have used is the result of several modifications to the above technique. The results of many experiments led to the adoption of the following procedure: Five samples were applied to column III of the amino acid analyzer at 10 minute intervals. The five peaks were eluted after two hours with the pH 5.28 buffer. As mentioned previously column III was an addition to the analyzer and was specifically designed for the determination of ammonia. A resin bed of 16 cm was chosen as this represented the best compromise between the number of samples which could be applied and the speed of the application. As a result five samples could be run on this column. If a sixth were added the first peak would be lost. More samples could have been applied to the column had a longer resin bed been used, but the loading time for each sample would have increased due to the higher back pressure.

a) Materials and Methods:

All the reagents used were analyzed to determine their ammonia content. In some cases several lots of compounds from different manufacturers had to be examined before a suitable one was found. The most troublesome

reagent was the sulfosalicylic acid used to precipitate the protein after an assay. In particular the ammonia levels in the substrates, present in high concentrations in the assay mixture, had to be especially low. Several recrystallizations of the amides were required before these were free of ammonia. The eluting buffer on the amino acid analyzer (0.35N, pH 5.28) was protected from atmospheric ammonia by means of a citric acid guard tube.

During the course of the literature search on the determination of ammonia, several papers led us to believe that the assays should be carried out at a pH of 7.50 or less. Above this pH, ammonia has the tendency to escape from solution (218). For this reason a pH of 7.50 was chosen. Since the enzyme requires the presence of calcium, the use of phosphate buffer was precluded. The most obvious next choice was tris (tris (hydroxymethyl) aminomethane) buffer, but this compound was found to be ninhydrin-positive. On column III of the analyzer tris gave a peak at 51.5 min. with a constant of 0.089; ammonia, on the other hand, came off the column at 71.5 min. Although the constant was very small the large amounts of buffer required made its use impracticable. Instead, a modified tris buffer TES (tris (hydroxymethyl) aminomethane sulfonic acid) was chosen instead because it was not ninhydrin-positive, and also because it has a pK

of 7.50 (223). TES was made up as a 0.02M stock solution containing 0.1M calcium chloride. This was diluted by one half in all the assay mixtures. The final mixture of buffer, calcium chloride, substrate and sulfosalicylic acid resulted in a blank of less than 0.1 optical density on the amino acid analyzer.

Standard curves for the determination of ammonia were run in the presence of buffer, calcium chloride and sulfosalicylic acid at the concentrations used in the enzyme assays. Ammonium sulfate (primary standard, Fisher cat. no. A-538) was used to prepare a stock solution of 6 μmoles/ml. A typical curve, consisting of eight points run in two segments, was determined as follows. A blank consisting of water (1.0 ml), buffer (1.0 ml), and 20% sulfosalicylic acid (1.0 ml) was prepared with each segment. Four samples containing from 0.1 to 0.8 ml (0.2 to 1.6 μmoles) of the stock solution made up with water to 1.0 ml were used as the standards. The first segment of the standard curve was run with a blank and 0.1 to 0.4 ml of the appropriate stock solution. The second segment was run with another blank and 0.5 to 0.8 ml of the stock solution. The curve was run in this way since only five samples could be run at one time, but at least eight points were desired on the curve. One milliliter of each of the above solutions were run on column III of the amino

acid analyzer as follows:

The blank was applied to the top of the column in the usual way, using compressed nitrogen and rinsing-in of the sample with the eluting buffer (3 x 0.2 ml). The top of the column was filled with the same buffer and the pump head secured. The column was pumped for 10 minutes to the drain, and at the end of this period the buffer was withdrawn from the top of the column and a second sample was applied as described. This process was repeated three more times. At the end of the fifth application the column effluent was sent to the reaction coil and the recorder turned on. The column was regenerated after use in the usual way, using 0.2N sodium hydroxide and re-equilibration, before the second set was run.

In this way a series of peaks was obtained; the data were handled in the following way: A plot of optical density versus concentration, which had yielded a straight line for amino acids (224), did not give satisfactory results for ammonia under these conditions. It was therefore found to be most convenient to use a graph of height-times-width versus concentration. A factor for conversion into μmoles/ml was calculated from the slope. The standard curve appears in Figure 11.

All enzyme incubations were carried out in small test tubes which were placed in a constant temperature bath

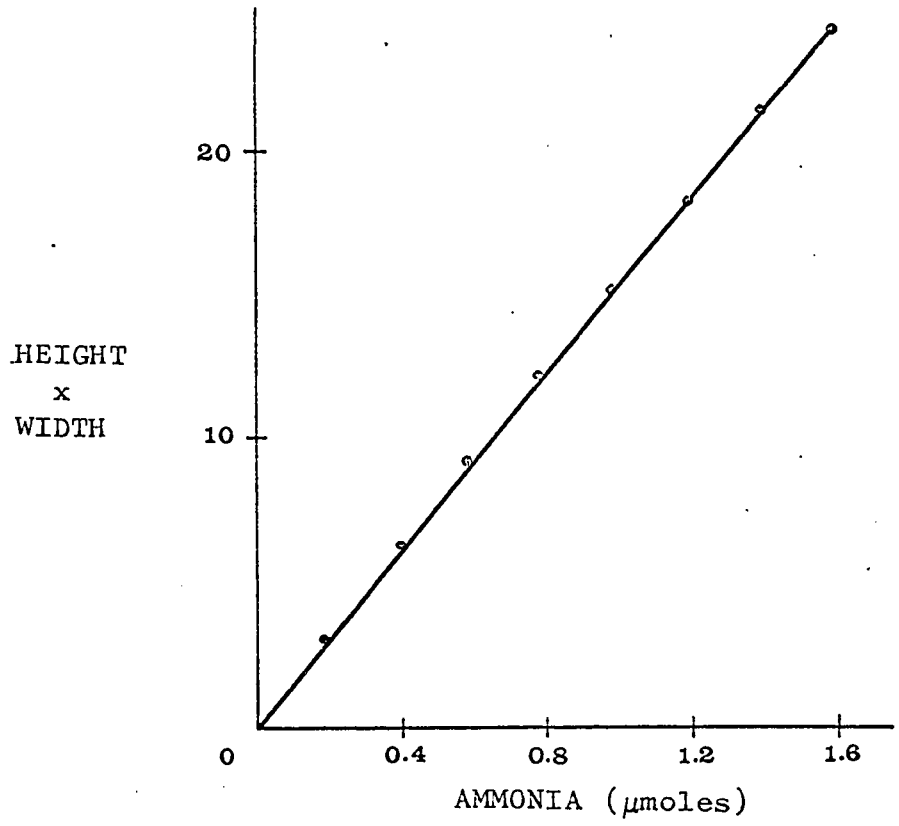


FIGURE 11 Standard curve for the determination of ammonia with the Beckman Amino Acid Analyzer.

at 25°, the solution being gently stirred with the aid of a magnetic stirrer.

Several enzyme and substrate blanks were carried out in order to ascertain whether there was any spontaneous ammonia production. The enzyme was dissolved in the buffer at the highest concentration used (4.2×10^{-4}M) and incubated for one hour. No ammonia production above the blank was detected after the addition of the sulfosalicylic acid. Another run using substrate alone at 0.1M also showed no spontaneous ammonia production.

As the hydrolysis of a substrate progressed, the amino acid amide was split into the amino acid and ammonia. In the case of α-N-benzoyl-L-lysinamide, for example, α-N-benzoyl-L-lysine and ammonia were produced. The α-N-benzoyl-L-lysine was eluted from the column very rapidly under the conditions used to measure ammonia. As a result the benzoyl lysine produced in the last sample applied had to traverse the ammonia peaks from previous applications on the column. In order to determine whether this would have an effect on the results, the following recovery experiment was run. Known amounts of ammonia were added to equal amounts of α-N-benzoyl-L-lysine in the presence of the corresponding amide, buffer, calcium chloride, etc. The tubes were incubated as if there were enzyme present, and 20% sulfosalicylic acid was added at regular

time intervals. The ratio of sulfosalicylic acid to reaction mixture was always 1:2. The results of a typical experiment appear in Table V.

Table V

Recovery of Ammonia in the Presence of Substrates and Products

time incubated (min)	amide (μmoles)	amino acid (μmoles)	ammonia added (μmoles)	ammonia found (μmoles)
0	5.0	0	0	0
10	5.0	0.30	0.30	0.29
20	5.0	0.60	0.60	0.61
30	5.0	0.90	0.90	0.93

The recovery was within ± 3% of the amount added.

The amino acid corresponding to the last two samples also appeared on the chromatogram. The first two were eluted from the column before the effluent was sent to the coil. In the case of the homolysine amide, where only three samples were applied and the amino acid emerged more slowly, it was possible to measure the production of both products. The results of a typical chromatogram are shown in Figure 12.

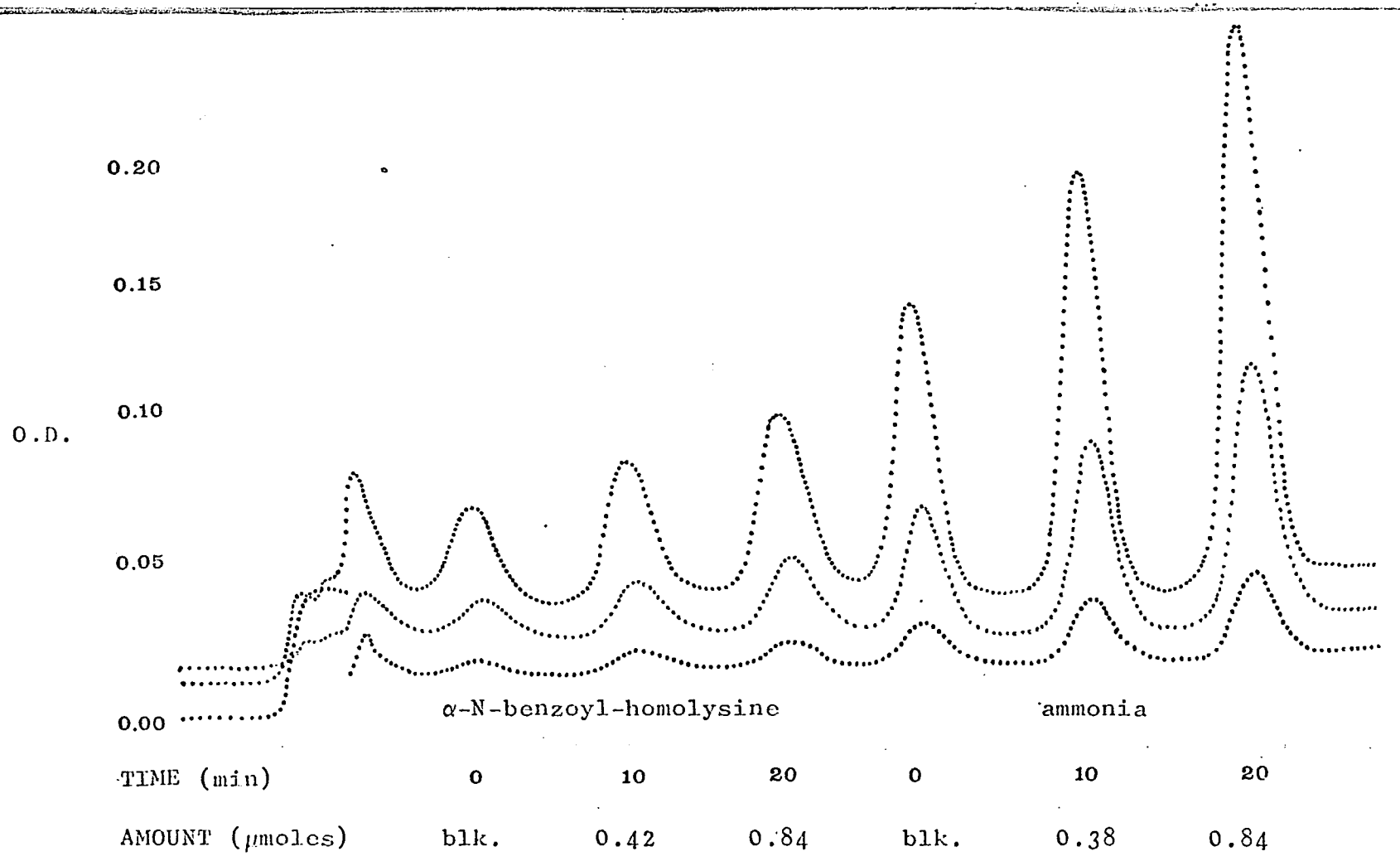


FIGURE 12 Hydrolysis of α-N-benzoyl-DL-homolysinamide by trypsin at pH 7.50 and 25° as obtained from the amino acid analyzer.

For the enzyme assays, one test tube was set up for each time interval to be studied. Four incubations, a blank and three reactions at regularly spaced time intervals, were usually carried out. The total volume of an assay mixture was from 0.75 to 3.0 ml, depending upon the rate of hydrolysis and the availability of the substrate. The enzyme-substrate ratio was always maintained at 1:100 at the minimum. The reactions were initiated by the addition of the enzyme and terminated by the addition of 20% sulfosalicylic acid. For the blank, sulfosalicylic acid was added before the enzyme. The assay tubes were cooled in a refrigerator for at least one half hour to ensure complete precipitation of the protein. The tubes were centrifuged (International Clinifuge) and an aliquot of the clear supernatant was run on the column as described previously. In this way a single rate curve was obtained. Several such runs were made at different substrate concentrations until at least six points were obtained in duplicate. The velocities were calculated so as to correspond to 1 liter and the data handled using the computer as for the esters. As before, the substrate concentration was varied between 0.5 and 5.0 times Km. This range was determined from the results of pilot experiments.

b) Hydrolysis of α-N-Benzoyl Amides by Trypsin

The following compounds were tested as substrates

as described above. α-N-Benzoyl-L-lysinamide was included for comparative purposes.

α-N-Benzoyl-L-lysinamide was hydrolyzed by trypsin at an appreciable rate. The results are plotted in Figure 13.

α-N-Benzoyl-ε-N-methyl-L-lysinamide was hydrolyzed slowly by trypsin. The kinetic data are plotted in Figure 14.

α-N-Benzoyl-ε-N,ε-N-dimethyl-L-lysinamide was not hydrolyzed by trypsin at substrate concentrations as high as 1×10^{-2}M and enzyme concentrations as high as 1.16×10^{-4}M.

α-N-Benzoyl-ε-N,ε-N,ε-N-trimethyl-L-lysinamide was not hydrolyzed by trypsin at substrate concentrations as high as 1×10^{-2}M and enzyme concentrations as high as 1.16×10^{-4}M.

α-N-Benzoyl-ε-N-formyl-L-lysinamide was not hydrolyzed by trypsin at substrate concentrations as high as 3×10^{-2}M and enzyme concentrations as high as 3.90×10^{-4}M.

α-N-Benzoyl-DL-homolysinamide was a substrate for trypsin. The kinetic data are plotted in Figure 15.

The kinetic data are summarized in Table VII.

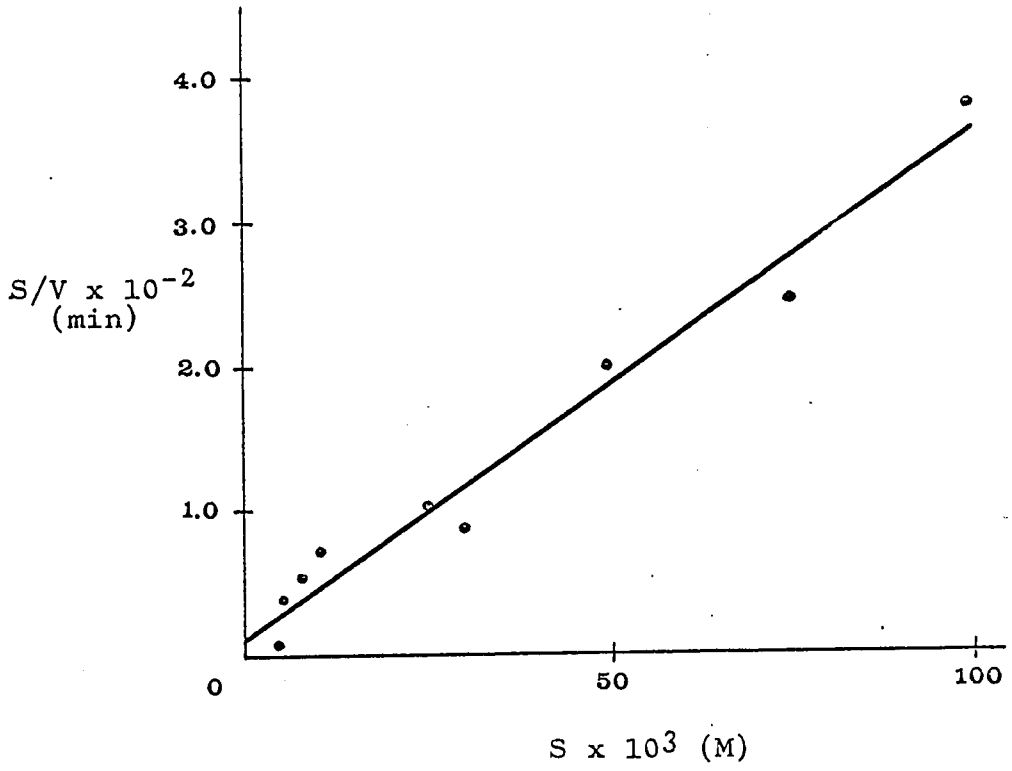


FIGURE 13 Hydrolysis of α-N-benzoyl-L-lysinamide by trypsin at pH 7.50 and 25°. [S] = 5 to 100 x 10^{-3}M; [E] = 2.25 x 10^{-5}M.

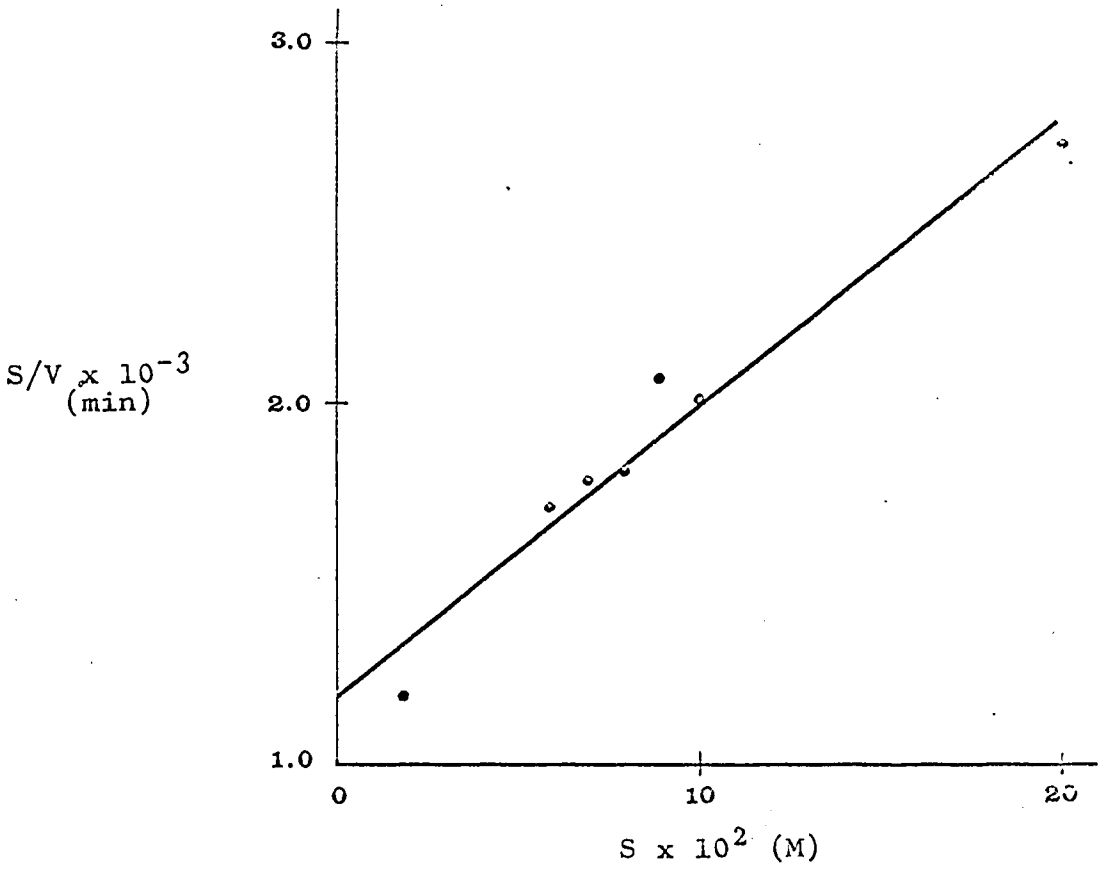


FIGURE 14 Hydrolysis of α-N-benzoyl-ε-N-methyl-L-lysinamide by trypsin at pH 7.50 and 25°. [S] = 2 to 20 x 10^{-2}M; [E] = 4.2 x 10^{-4}M.

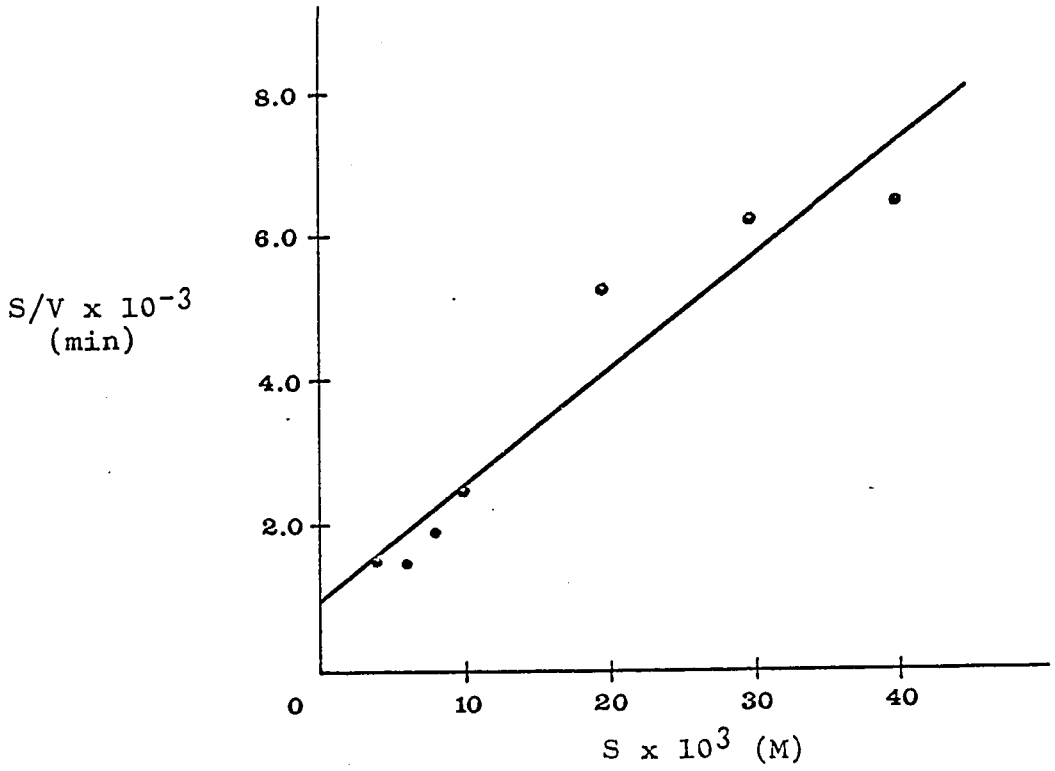


FIGURE 15 Hydrolysis of α-N-benzoyl-DL-homolysinamide by trypsin at pH 7.50 and 25°.
[S] based on L isomer = 4 to 40 x 10^{-3}M;
[E] = 4.73 x 10^{-5}M.

Table VII

Kinetic Data for the Hydrolysis α-N-Benzoyl Amides by Trypsin at pH 7.50 and 25°

Compound	Km(app) (moles l^{-1})	Vmax (moles min^{-1})	k_{cat} (min^{-1})
α-N-Benzoyl-L-lysinamide	4.25×10^{-3}	2.92×10^{-4}	13.0
α-N-Benzoyl-ε-N-methyl-L-lysinamide	162.0×10^{-3}	1.22×10^{-4}	0.29
α-N-Benzoyl-ε-N,ε-N-dimethyl-L-lysinamide	not a substrate		
α-N-Benzoyl-ε-N,ε-N,ε-N-trimethyl-L-lysinamide	not a substrate		
α-N-Benzoyl-ε-N-formyl-L-lysinamide	not a substrate		
α-N-Benzoyl-DL-homolysinamide	6.50×10^{-3}	6.3×10^{-6}	0.15

3. Hydrolysis of Poly-L-lysines by Trypsin:

Katchalski (61) found tht crystalline trypsin readily attacked poly-L-lysine, and showed that about half the peptide bonds were split. It was shown that no free lysine was formed. Waley and Watson (58) investigated the hydrolysis using a pH-stat; they followed the formation of products with thin layer chromatography. We have studied the hydrolysis of poly ε-N-methyl-L-lysine using similar methods.

The poly-ε-N-methyl-L-lysine we had prepared was checked for purity using thin layer chromatography on cellulose plates (ascending technique) with the solvent system described by Waley and Watson (n-butanol: acetic acid: water: pyridine, 30:6:24:20, V/V). The polymer was chromatographed against standards of lysine and ε-N-methyl-L-lysine. Also included were di-, tri-, tetra-, and pentalysine (Sigma, St. Louis, Mo.; donated by Dr. P.S. Fitt). The results of this chromatogram are illustrated in Figure 16a. This shows that the lower the degree of polymerization the greater the mobility of the compound. Our polymer was therefore free of any low-molecular-weight contaminants.

A sample of the poly-ε-N-methyl-L-lysine (50 mg) was dissolved in 0.1M TES buffer, pH 7.50 (1.0 ml), as described previously for the amides. Crystalline trypsin

(2 mg/100λ 0.001N HCl) was added and the mixture was incubated for 12 hours at 25°C. At the end of this period samples were chromatographed in the same solvent system described above. The unhydrolyzed polymer and both lysine and ε-N-methyl-L-lysine were run as markers. The results of this chromatogram are illustrated in Figure 16b. This shows that poly-ε-N-methyl-L-lysine was hydrolyzed by trypsin and that no free ε-N-methyl-L-lysine was formed. This is in good agreement with the results found for poly-L-lysine where only intermediate-sized peptides are formed.

The rate of hydrolysis of the poly-ε-N-methyl-L-lysine was compared to the rate observed with poly-L-lysine on the pH-stat using the technique described previously for the esters. The sole exceptions were that the ionic strength was 0.05M in calcium chloride and that the pH was 7.50, as for the rest of the amides. The polymers of L-lysine and ε-N-methyl-L-lysine were used at a concentration of 50 mg/ml, with an enzyme concentration of 0.025 mg/ml. The rate of hydrolysis of the poly ε-N-methyl-L-lysine under these conditions was about 80% of that of poly-L-lysine. Since we only had molecular weight data for our poly ε-N-methyl-L-lysine the experiment was repeated once using a poly-L-lysine of molecular weight 2600

FIGURE 16b Chromatography of poly-ε-N-methyl-L-lysine after incubation with trypsin.

FIGURE 16a Chromatography of oligolysine peptides and poly-ε-N-methyl-L-lysine.

(Sigma, St. Louis, Mo.; donated by Dr. P.S. Fitt). This compared favorably with the molecular weight of our polymer (2240) and gave an identical rate of hydrolysis to that of our poly-L-lysine.

4. Discussion

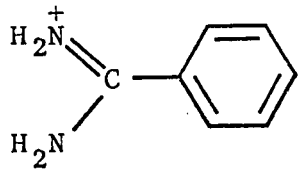
Until recently it was believed that substitution on the ε-amino group of a lysine ester or amide would entirely prevent hydrolysis (47). All previous work with small model compounds, such as those shown in Table I, as well as with substituted proteins, had indicated that this was true. The evidence for the latter rested upon the observation that acyl (225,226), carbamyl (227), carboxymethyl (228), or acetimidyl (229) substituted lysine residues were resistant to tryptic hydrolysis. Furthermore, even when an ε-nitrogen was substituted with an alkyl group, dinitrophenyl, there was also no hydrolysis (230). The discovery by Benoiton and Deneault (26) that two ε-N-methyl-L-lysine derivatives were substrates was therefore unexpected. These results were explained when it was observed that all previous substituents on the ε-amino group were such as to eliminate the positive charge from this nitrogen. This was the case even for the dinitrophenyl derivative which is only protonated in strongly acidic conditions. Where the methyl group was used as the substituent, the positive charge was not eliminated, and the hydrolysis took place. This observation was not supported by the discovery that an ε-N-isopropyl residue in reduced acetoacetate decarboxylase was unaffected by trypsin. The trypsin concentration used for the hydrolysis

was lower than would be required even for an ε-N-methyl lysine residue to be split (54).

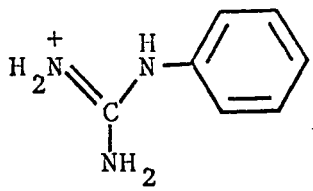
The discovery by Kloss and Schröder (50) that ε-N-formylated as well as ε-N-tosylated lysines in peptide esters were hydrolyzed by trypsin, despite the lack of a positive charge, raised some doubts as to the validity of the proposed theories. Before our results are discussed in detail, the following points about the mechanism of action of trypsin should be considered.

As mentioned previously, the studies of Jansen, Nutting, Jang and Balls (125) with diisopropyl phosphofluoridate indicated that one serine residue of trypsin was especially reactive. The studies of Gutfreund (80) on the pH dependence of the trypsin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester indicated that a histidyl residue participates in the reaction. Similarly, reagents such as the chloromethyl-ketone derived from α-N-tosyl-L-lysine (TLCK) provided further evidence that a histidine residue participates in the catalytic process. As a consequence of their studies on the binding of amidines and guanidines to trypsin, Mares-Guia and Shaw (231) proposed that the term "specificity site" be used to encompass the catalytic site as well as the anionic site. The binding of these compounds was studied by measuring their inhibition of the hydrolysis of α-N-benzoyl-DL-arginine p-nitroanilide (DL-BANA). In order to do this it was necessary to include

the contribution of a second inhibitor, viz., the D-isomer of the nitroanilide. Amidines and guanidines were selected for these studies because, besides fulfilling one of the specificity requirements of trypsin, viz., having a positive charge, they also had model "side chains" which had a limited number of sterically different structures. Two of the compounds used for these studies are shown below:



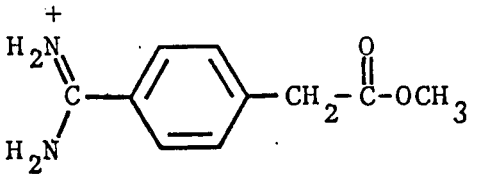
benzamidine



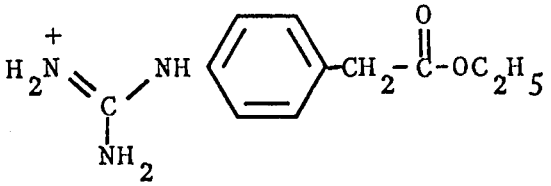
phenylguanidine

This type of compound was found to inhibit the hydrolysis of DL-BANA and was therefore bound to the enzyme. On the basis of these results, it was suggested that the charged portion of the inhibitor bound to the anionic site and the hydrophobic part of the molecule bound to a hydrophobic site between the anionic site and the catalytic site. These experiments did not prove that the hydrophobic binding area had to be located as described. The results could equally well have been explained if the hydrophobic region were elsewhere. Mares-Guia and Shaw solved this problem by showing that ester derivatives of the above

inhibitors were hydrolyzed by the enzyme (232). They found that compounds such as methyl p-amidinophenylacetate and ethyl p-guanidinophenylacetate, in which the distance between the positive charge and the carbonyl group is similar to that of the side chain of fully extended normal substrates, are hydrolyzed quite readily. These compounds are shown below:

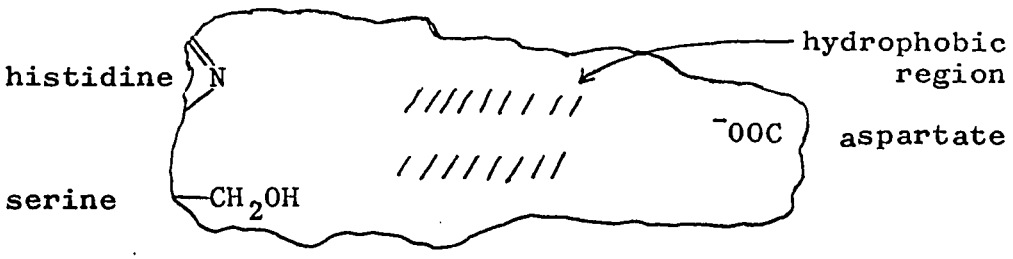


methyl p-amidinophenyl-acetate



ethyl p-guanidinophenyl-acetate

On the basis of these results, the normal function of this hydrophobic region was considered to be the binding of the methylene groups in lysine and arginine substrates. Further elucidation of the structure of the specificity site has come from the work of Blow (233). He has found that at the bottom of this hydrophobic area or "hole" there is the carboxyl group of Asp 177. This residue was previously Asn 177. Thus the major components in the specificity site may be represented as follows:



Specificity Site of Trypsin

There is evidence for an auxiliary binding site on trypsin which can accomodate both positively charged (120) and neutral (126) molecules. These observations were confirmed by the work of Sanborn and Hein (30). These authors found, for example, that phenylurea, which is a neutral molecule, inhibits the hydrolysis of α-N-benzoyl-L-citrulline methyl ester, which is also neutral. However, phenylurea does not inhibit the hydrolysis of α-N-benzoyl-L-arginine methyl ester which is a charged substrate. These results would suggest that there is a binding site specific for neutral molecules of this kind. This second hydrophobic site must be near the catalytic site, since the hydrolysis of the neutral substrates proceeds by the same mechanisms. There must also be some overlap in the binding sites since phenylurea has no effect on acetyllysine methyl ester, but does on benzoylglycyl lysine methyl ester.

In an attempt to clarify the relationship between the charge on the amino group, the substituent on the group, and the susceptibility of these compounds to trypsin, a series of ε-N-mono-, di-, and trimethyl as well as two ε-N-formyl lysines was prepared.

a) Methyl lysines

The studies of Benoiton and Deneault (26) on ε-N-methyl-L-lysine ethyl ester were only of a semi-quantitative nature. The relative rate of hydrolysis of this substrate

was compared with that of L-lysine ethyl ester, by measuring the disappearance of the ester by the method of Hestrin (234). When this experiment was carried out at substrate concentrations of 2.0×10^{-2}M and at pH 8.0 a rate of 3200 μmoles/hr/mg enzyme was found for the non-methylated ester, and 194 μmoles for the methylated ester (17 to 1). Before undertaking detailed kinetic work with these substrates, we verified the results of this experiment using purified enzyme (Worthington). In this case the rate of appearance of product was measured using the amino acid analyzer. Under the conditions described by Benoiton and Deneault we found a rate of 3100 μmoles/hr/mg enzyme for L-lysine ethyl ester and 86.2 μmoles/hr/mg enzyme for the corresponding ε-N-methyl ester (36 to 1). The source of the discrepancy between our results and theirs was at first attributed to the fact that they had used enzyme purchased from a different supplier (Nutritional Biochemicals). However, when the experiment was repeated with enzyme obtained from Nutritional Biochemicals, a rate of 87.6 μmoles/hr/mg enzyme was found. It is possible to explain these results if one allows either for transpeptidation or for a L-lysine ethyl ester contaminant in the ε-N-methyl-L-lysine ester used by Benoiton and Deneault. An analysis of our kinetic data shows that at the pH optimum (6.2) the ratio of the

rates of hydrolysis for the two esters is 1 to 19. It is only for α-N-acyl lysines that the pH optimum is 8.0.

The three monoalkylated derivatives (ε-N-methyl-L-lysine ethyl ester; α-N-benzoyl-ε-N-methyl-L-lysine methyl ester and its corresponding amide) were the only methyl lysines hydrolyzed by trypsin. All of the di-(ε-N,ε-N-dimethyl-L-lysine ethyl ester; α-N-benzoyl-ε-N, ε-N-dimethyl-L-lysine methyl ester and its corresponding amide) and tri-(α-N-benzoyl-ε-N,ε-N,ε-N-trimethyl-L-lysine methyl ester and its corresponding amide) methyl lysines were entirely resistant to trypsin.

Throughout the kinetic work the corresponding non-methylated lysine derivatives were run in parallel as controls. The data obtained with these substrates are compared with the values found for similar substrates by other workers.

	Found		Literature		
compound	Km (app) moles l^{-1}	k_{cat} min^{-1}	Km (app) moles l^{-1}	k_{cat} min^{-1}	Ref.
Lys-OEt	1.82×10^{-3}	878	9.3×10^{-3}▲	820	24
Bz-Lys-OMe	2.78×10^{-5}	519	5.5×10^{-5}	504*	30,24*
Bz-Lys-NH_2	4.25×10^{-3}	13	4.6×10^{-3}	39*	35,102*

* given for the corresponding arginine derivative

▲ pH 8.0

The dissociation constants for all of the amino acid amides tested were measured as previously described. The results obtained parallel those found when other amino groups are similarly substituted e.g. glycine, sarcosine and dimethyl glycine (cf 168). Our value for α-N-benzoyl-L-lysinamide is 0.6 pK unit lower than that found by Wang and Carpenter (35). A summary of the data obtained appears below:

Compound	pK	Km (app) moles l^{-1}	k_{cat} min^{-1}
Bz-lys·NH_2	9.69	4.25×10^{-3}	13.0
Bz-lys(Me)-NH_2	10.41	162.0×10^{-3}	0.29
Bz-lys(Me,Me)-NH_2	9.53	not a substrate	
Bz-lys(Me,Me,Me)-NH_2	---	not a substrate	

These results show that there is no relationship between the charge and basicity of the compound and its susceptibility to the enzyme.

The failure of any of our ε-N,ε-N-dimethyl derivatives to be hydrolyzed was confirmed by the results of Gorecki and Shalitin, which appeared during the course of this work (24). They found that α-N-acetyl-ε-N,ε-N-dimethyl-L-lysine methyl ester was not hydrolyzed by trypsin. These results are quite surprising at first

sight since there is a close structural similarity between ε-N,ε-N-dimethyl-L-lysine and L-arginine. The dimethyl lysine has a nitrogen atom surrounded by three carbon atoms, while in arginine the guanidinum carbon atom is surrounded by three nitrogen atoms. However, the charge on the arginine is localized of the nitrogens at the end of the side chain. When an arginine substrate is bound to the enzyme no group protrudes beyond the anionic site. In the case of ε-N,ε-N-dimethyl-L-lysines, however, the two methyl groups will lie beyond the anionic site if bound. Gorecki and Shalitin had explained their results on the basis that the dimethyl lysine lacked a hydrogen-bondable atom at the cationic group, and thus could not be bound.

The observation that both the ε-N-dimethyl and ε-N-trimethyl lysines failed to inhibit the hydrolysis of α-N-benzoyl-L-lysine methyl ester was unexpected, since other charged compounds affect the enzyme (231,232). This would suggest these compounds are not bound to the enzyme.

The ε-N-monomethyl lysine derivatives are hydrolyzed quite slowly by trypsin. The k_{cat} values are of the same order of magnitude as those of unalkylated lysines. Since the deacylation occurs at about the same rate, the binding of the methylated derivative must be poorer. This decrease could once again result from the fact that there

is an additional residue protruding beyond the anionic site when the substrate is bound. That this phenomenon was not due solely to the increased length of the side chain of the molecule is shown by the ease with which homolysine is hydrolyzed. The methyl group may cause poorer binding because hydrophobic considerations prevent the group from going past the anionic site. It would be interesting to study the effect of a bulkier and more hydrophobic group (eg. isopropyl) on the kinetics.

b) Polymers

Of particular interest was the action of trypsin on poly-ε-N-methyl-L-lysine. The synthesis of the polymer was straightforward but one molecule of water per residue had to be included in order to satisfy the elemental analysis. It could have been suggested that the product obtained was not a polymer at all, but both the molecular weight determination and the chromatography of this compound showed that such was not the case. Furthermore, the infrared spectrum was characteristic of a polymerized amino acid. It is known that poly-L-lysine·HBr is similarly hygroscopic.

The observation that the poly-ε-N-methyl-L-lysine was hydrolyzed at 80% of the rate of poly-L-lysine was not expected, in view of the difference found in the

kinetics of α-N-benzoyl-ε-N-methyl-L-lysinamide and α-N-benzoyl-L-lysinamide. However, in view of the fact that the rates of hydrolysis of the polymers were carried out using the pH-stat, an absolute interpretation of these rates is difficult. This is due primarily to the fact that, as the hydrolysis progresses, an α-amino group as well as a proton are released at each point of cleavage. Since the liberated α-amino groups of the lysine and ε-N-methyl-L-lysine are likely to have different pK values, it is difficult to calculate the relative rates of hydrolysis. Thus one amino group may have a greater buffering capacity than the other, so that the actual amount of the hydrolysis actually detected by the pH-stat is uncertain. The rate of hydrolysis could be more accurately measured by monitoring the production of products from the polymers. For example, the amino acid analyzer could be used to measure di- and trilysine production. The most immediate difficulty in such an approach is the lack of methylated standards such as ε-N-methyl-L-lysyl-(ε-N-methyl)-L-lysine.

The results of the experiments on methylated lysines are especially interesting in view of the attention which is currently being focused on ε-N-methylated lysines. Trypsin has long been used, because of its restricted specificity, to fragment proteins into smaller peptides for sequence work. Since methyl lysines are being found in proteins,

a knowledge of the specificity of trypsin towards these residues is invaluable to anyone contemplating such sequence work. To [illegible], ε-N-methyl-L-lysine has been found to occur in flagellar proteins (235), ε-N-mono, di, and trimethyl-L-lysines in histones (236-238) and more recently ε-N,ε-N,ε-N-trimethyl-L-lysine in cytochrome C (239). Furthermore, the results with poly ε-N-methyl-L-lysine indicate that when such residues occur in proteins the rate of hydrolysis by trypsin may be almost as rapid as at a lysine residue.

c) Formyl lysines

Neither of the two formyl derivatives (ε-N-formyl-L-lysine methyl ester, α-N-benzoyl-ε-N-formyl-L-lysinamide) were hydrolyzed by trypsin. Both of these substrates were water-soluble. As mentioned previously, the work of Kloss and Schröder (50) showed that the presence of a formyl or a tosyl group on the ε-amino nitrogen did not always prevent hydrolysis. Most of the 51 peptides which were tested as trypsin substrates were segments originally used in the synthesis of eledoisin by one of the aformentioned authors (240). Many of the compounds tested were N-carbobenzoxy peptides and thus were not water-soluble. Kloss and Schröder circumvented this problem by resorting to the technique described by Walton et al. (154), who used the chymotryptic hydrolysis of substrates

dissolved in dimethylformamide as a preparative method for the synthesis of angiotensin analogues. This was accomplished by dissolving a peptide ester in dimethylformamide and adding this dropwise to a stirred solution of the enzyme at the pH desired. The initial enzyme-substrate ratio used was from 1:1000 to 1:100,000. In the former case, if the substrate solution were added in ten drops, the enzyme-substrate ratio at any given amount would be 1:100, which is the generally accepted maximum. In the paper of Kloss and Schröder, however, the initial enzyme-substrate ratio used was 1:100. If one once again assumes a 10-drop addition of substrate as described above, an enzyme-substrate ratio of more than 1:10 would be possible at any given moment. Under these circumstances, the results of any hydrolysis observed are questionable because the presence of small amounts of another contaminating enzyme in the trypsin might become significant. Unfortunately, the exact experimental conditions used in the experiments of Kloss and Schröder (eg. substrate concentrations) were not clearly specified. Only the total time of incubation of enzyme and substrate are given without any indication of the rate of hydrolysis. In some cases the authors extracted the product of the hydrolysis into an organic solvent, hydrogenated, treated with leucine aminopeptidase, and chromatographed the resulting digest.

The chromatogram for Z-Pro-Ser-Lys(For)-OMe (# 65), for example contained proline, serine and ε-N-formyl-L-lysine, indicating that the hydrolysis had occured originally at the ester residue. The hydrolysis may thus have been due to (a) a high enzyme-substrate ratio or (b) the action of the organic solvent on the enzyme.

Another explanation of the whole phenomenon may lie in the findings of Coletti-Previero et al. (239). These investigators have been able to separate the esterasic and proteolytic activities of trypsin by formylating the tryptophan residues of the enzyme. Trypsin so treated was not active on a model amide (DL-BANA) nor on a protein (casein), but retained activity towards α-N-benzoyl-L-arginine ethyl ester. It was also observed that the same behaviour could be duplicated by using the enzyme in 50% aqueous dioxane, 25% aqueous formamide or 37% aqueous 2-chloroethanol. Since the mechanism of hydrolysis of amides and esters is the same, it is difficult to understand these results unless one assumes that they are due to an artefact. The conformational changes caused by solvents or formylation may produce an "esterase" which is no longer specific for normal substrates. This would readily explain the results of Kloss and Schröder.

d) Homologues

The action of trypsin on the derivatives of lysine

homologues with the exception of several ornithine compounds (34,116) had not been investigated. Elmore et al. (116,131) have investigated the effect of chain length on kinetic parameters for substrates ranging from α-amino-δ-guanidinobutyric acid to homoarginine. The higher and lower homologue derivatives of these compounds are hydrolyzed, with the exception of the shortest, α-amino-δ-guanidino-butyric acid. The rates of hydrolysis of the homologues are markedly lower than those of the corresponding arginine derivative. The upper limit for chain length had not been investigated. In order to see whether the failure of ε-N-methyl-L-lysines to be good substrates was due to the total length of the chain we prepared the next two homologues of lysine, viz., 2,7-diaminoheptanoic acid (homolysine) and 2,8-diamino-octanoic acid.

The homolysine derivatives (DL-homolysine ethyl ester, α-N-benzoyl-DL-homolysine methyl ester and its corresponding amide) were all found to be good substrates for trypsin despite the fact that the racemate was used. The results of Kitagawa and Izumiya (94) with the benzoyl amides of DL- as well as L-arginine show that, while both are hydrolyzed by the enzyme, the rate of hydrolysis of the racemate is only 60% of that of the L-isomer. This is probably due to the fact that the D-isomers of such

derivatives are bound and act as competitive inhibitors (30,231,242). Thus it seems reasonable to suggest that if the pure L-isomers of the homolysines tested were available, the kinetic parameters would be even closer to those of lysine derivatives.

As mentioned previously, it was possible to obtain D-homolysine in a relatively pure form. A sample of this "D"-isomer (10% L-isomer) was esterified exactly by the same method as the racemate. The hydrolysis of this product was followed on the pH-stat as previously described. The alkali consumption continued beyond the value representing 10% hydrolysis, suggesting that the D-isomer was being hydrolyzed. This would explain the difficulties encountered in the attempted resolution of DL-homolysine ethyl ester using trypsin. The fact that the D-isomer is hydrolyzed was quite unexpected, since this is not the case for chymotrypsin (193). We were unable to find any studies on the action of trypsin on DL-lysine ethyl ester or similar compounds. There are very few data on the action of trypsin on α-N-acyl-D-esters. In two studies (30,116) the enzyme appears not to be entirely stereospecific. This problem warrants further investigation.

The diaminoöctanoic acid ethyl ester was completely resistant to trypsin. This can readily be

explained in terms of our model for the specificity site. It is in the nature of the hydrophobic site to bind and keep the side chains of substrates fully extended (232,233). In this case the charged ω-amino group would not be in a very good position to bind properly with the anionic site.

Baird, Curragh and Elmore (131) were the first to try to relate kinetic parameters to chain length. By assuming that the positive charge on both arginine and lysine substrates is localized about the amino group, we have made the following observation:

The distances between the α carbon atom and the nitrogen bearing the positive charge (measured on scale models; Buchi, Switzerland) were plotted against the Km values observed for several α-N-acyl ester derivatives as shown in Figure 17. This diagram indicates that the best "binding" occurs when the chain is approximately 7Å long; the distance estimated to exist between the catalytic site and the anionic site (Asp 177).

One of the more useful methods of determining the relative ease with which substrates are hydrolyzed by an enzyme is the calculation of the ratio k_{cat}/Km (243). Table VII, below, gives values obtained for our substrates.

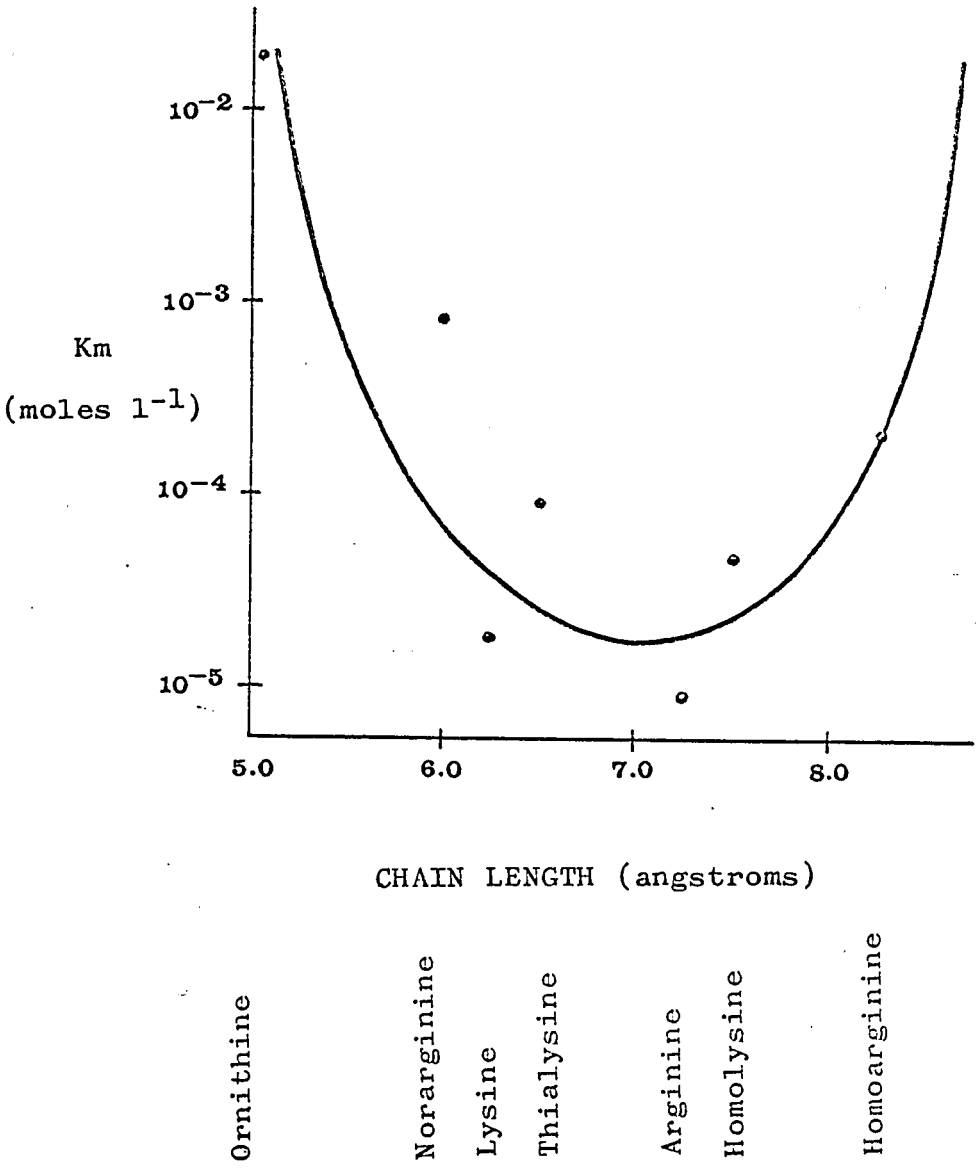


FIGURE 17 Plot of Km versus length of side chain for some α-N-acyl esters of basic amino acids.

Table VII

Comparison of k_{cat}/Km Values for Synthetic Substrates

Compound	k_{cat}/Km 1 $mole^{-1}$ min^{-1}
L-Lysine Ethyl Ester	4.8×10^6
α-N-Benzoyl-L-lysine Methyl Ester	9.0×10^8
α-N-Benzoyl-L-lysinamide	3.1×10^3
ε-N-Methyl-L-lysine Ethyl Ester	1.4×10^3
α-N-Benzoyl-ε-N-Methyl-L-lysine Methyl Ester	5.9×10^4
α-N-Benzoyl-ε-N-Methyl-L-lysinamide	1.8
DL-Homolysine Ethyl Ester	1.1×10^5
α-N-Benzoyl-DL-homolysine Methyl Ester	6.7×10^5
α-N-Benzoyl-DL-homolysinamide	2.3×10

Lysine and arginine derivatives are the best substrates known for trypsin. The above data show that aside from α-N-benzoyl-L-thialysine methyl ester, which gives a k_{cat}/Km ratio of 1.3×10^7 (31), the second best substrates are those of homolysine derivatives. The value for α-N-losyl-L-homoarginine is considerably lower (3.3×10^4; 116).

Summary

An investigation into some aspects of the behaviour of trypsin towards substituted lysines, with respect both to the nature of the substituent and to the charge on the ε-amino nitrogen, led to the preparation of several new compounds which were tested as substrates for the enzyme.

The compounds synthesized were based on three model substrates: L-lysine ethyl ester, α-N-benzoyl-L-lysine methyl ester, and α-N-benzoyl-L-lysinamide. a) A series of lysine derivatives having from one to three methyl groups on the ε-amino nitrogen were synthesized. This also included the preparation of poly-ε-N-methyl-L-lysine. b) Two compounds bearing a formyl group on the ε-amino nitrogen were prepared. c) The two higher homologues of lysine as well as a number of derivatives were prepared.

The compounds were incubated with trypsin and the rates of hydrolysis, if any, were measured in one of two ways: a) proton release on the pH-stat for esters and polymers, and b) ammonia release on the Amino Acid Analyzer for amides. An improved method for measuring the ammonia produced by enzyme reactions is described. The Km and k_{cat} values were measured for those compounds which are substrates. The actions of ε-lysine acylase from hog and chicken, L-lysine decarboxylase, L-amino acid oxidase,

and renal acylase I on the appropriate homolysine derivatives are described.

The results are as follows: a) of the ε-N-methylated derivatives only monomethyl-L-lysines are hydrolyzed. b) ε-N-formyl-L-lysine derivatives of our compounds are not hydrolyzed by trypsin. c) homolysine derivatives are excellent substrates for trypsin and d) poly ε-N-methyl-L-lysine is hydrolyzed by trypsin at an appreciable rate. These results are interpreted in terms of the proposed mechanisms of trypsin hydrolysis.

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