I. STEREOSPECIFICITY IN ALPHA-CHYMOTRYPSIN-CATALYZED REACTIONS

II. THE STRUCTURAL SPECIFICITY OF ALPHA-CHYMOTRYPSIN: SOME NEW SUBSTRATES

III. A FURTHER STUDY OF MONOFUNCTIONAL AROMATIC INHIBITORS OF ALPHA-CHYMOTRYPSIN

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ABSTRACT

A series of acylated glycine, D- and L-alanine methyl esters have been evaluated as substrates for alpha-chymotrypsin. These results are correlated with a theory concerning the stereospecificity of the enzyme.

The effect on the kinetic constants of reducing an aromatic substrate to its hydroaromatic counterpart is studied. Two classes of substrates are distinguished, one in which the ring is in the side chain and the other where the ring is part of the acylamido group.

Several analogues of known substrates are examined and their relationship and importance to the general picture of the specificity of alpha-chymotrypsin are discussed.

A series of simple aromatic inhibitors of alpha-chymotrypsin were examined. Concomitantly, the combined effect of two inhibitors on alpha-chymotrypsin-catalyzed hydrolyses is discussed.

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PART I

STEREOSPECIFICITY IN ALPHA-CHYMOTRYPSIN-CATALYZED REACTIONS

A. INTRODUCTION

In an attempt to understand the stereo- and structural specificity of the α-chymotrypsin-catalyzed hydrolysis of trifunctional substrates, of the general formula $R_1'CONHCHR_2COR_3$, where $R_1'CONH = R_1$, and $R_2 \neq H$, Hein and Niemann (1a,b,c) developed a general theory of enzyme specificity. This theory was subsequently utilized by Wolf and Niemann (2a,b) to explain the specificity observed in the alpha-chymotrypsin-catalyzed hydrolysis of acylated glycine derivatives, or bifunctional substrates of the general formula $R_1'CONHCH_2COR_3$. It was realized that while bifunctional substrates were handily accounted for in terms of the above theory, they offered more ambiguity than the trifunctional substrates and, hence, were accounted for in a much more qualitative manner. The work herein described was initiated to bring the picture of structural specificity for the bifunctional substrates into sharper focus. It was soon realized that by working with acylated alanine derivatives, the simplest trifunctional substrates, a deeper understanding of stereospecificity and structural specificity in both bi- and trifunctional substrates could be developed.

B. RESULTS

The kinetic procedure has been extensively described in the literature (3). All kinetic experiments were performed in aqueous solution at 25.0 ± .1° and pH 7.90 ± .05. The reactions were followed by means of a pH-stat (4) which gave primary data in the form of a recorder trace. Initial velocities were computed from these recorder traces by the orthogonal polynomial procedure of Booman and Niemann (5) which was programmed for the Datatron 220 computer by Abrash (6). The kinetic parameters K_o and k_o (see equation 1) were obtained by a least squares procedure with the aid of the above-mentioned program. All enzyme catalyzed reactions appeared to be described by the following rate expression:

$$-\frac{d(S)}{dt} = \frac{d(P)}{dt} = \frac{k_o(E)\ (S)}{(S) + K_o} \qquad (1)$$

The results are summarized in Table 1.

Table I

The α-Chymotrypsin-catalyzed Hydrolysis of a Series of Acylated Amino Acid Esters Containing Heterocyclic Acyl Moieties[a]

Substrate	$(S)_o$, mM	$(E)_o$[b], M	No. of[c] expts.	K_o, mM	k_o, $sec.^{-1}$
Methyl picolinurate	3.3-49	3.6	18-1	11.6±.9	2.02±.06
Methyl picolinurate[d]	3.3-26	3.5	11-0	10 ± 1	2.46±.07
Methyl-2-quinolinurate	.21-1.7	36	12-0	.35±.05	.0067±.0003
2-Thiophenoyl glycine methyl ester	.88-18	6.6	13-0	3.1 ±.1	.222±.003
o-Amino methyl hippurate	.49-3.9	39	14-1	2.4 ±.2	.35 ± .01
Methyl tetrahydrofurourate	18-143	37	11-0	86 ± 10	.143±.007
Isonicotinoyl-L-alanine methyl ester	5.6-45	29	11-0	29 ± 3	.42 ±.03
Isonicotinoyl-D-alanine methyl ester	4.2-33	57	8-1	27 ± 9	.003±.001
Nicotinoyl-L-alanine methyl ester	2.6-21	37	15-0	37 ± 2	.58 ±.02
Nicotinoyl-D-alanine methyl ester	2.7-22	71	13-0	6 ± 1	.0046±.0002
Picolinoyl-L-alanine methyl ester	2.3-18	22	10-0	18 ± 1	.071 ±.003
Picolinoyl-D-alanine methyl ester	1.6-13	63	8-0	17 ± 1	0.168 ±.006
Furoyl-L-alanine methyl ester	12-99	34	8-1	49 ± 3	.51 ± .01
Furoyl-D-alanine methyl ester	3.1-25	38	11-0	19 ± 3	.039±.003

Table I (Cont'd)

Substrate	$(S)_o$,mM	$(E)_o$, M	No. of expts.	K_o,mM	k_o,sec.$^{-1}$
Tetrahydrofuroyl-L-alanine methyl ester	20-201	43	14-0	132±15	.40± .03
Tetrahydrofuroyl-D-alanine methyl ester	10-80	56	10-0	61±10	.017±.002
2-Thiophenoyl-L-alanine methyl ester	2.2-18	45	11-0	15± 4	.46 ±.06
2-Thiophenoyl-D-alanine methyl ester	1.9-15	45	9-1	7.4±.6	.0159±.0006
o-Aminobenzoyl-L-alanine methyl ester	.68-5.5	37	16-0	4.7±.7	.24 ± .02
o-Aminobenzoyl-D-alanine methyl ester	.66-5.3	44	12-0	1.6±.5	.0050±.0005
2-Quinolinoyl-L-alanine methyl ester	.16-1.3	48	12-0	.22±.17	.0040±.0007
2-Quinolinoyl-D-alanine methyl ester	.80	48	Not a substrate[e]		
Nicotinoyl-L-valine methyl ester	2.1-17	24	11-0	5.7±.7	.053 ±.003
Picolinoyl-L-valine methyl ester	.97-7.7	81	11-1	10 ± 2	.029 ±.004
Picolinoyl-D-valine methyl ester	7.9	81	Not a substrate[e]		
Furoyl-L-valine methyl ester	2.0-16	24	10-2	19 ± 1	.157 ±.006
L- -Picolinamido methyl-n-butyrate	1.2-9.7	24	12-1	9.0±.6	.36 ± .01
Picolinoyl-L-norvaline methyl ester	.56-4.5	2.5	16-1	5 ± 1	1.9 ± .3
Furoyl-L-phenylalanine methyl ester	.21-1.9	3.2×10^{-3}	13-2	.36±.09	74 ± 5
Tetrahydrofuroyl-L-phenylalanine methyl ester	.19-1.7	3.2×10^{-3}	13-0	1.2±.4	95 ± 19

Table I (Cont'd)

a) In aqueous solution at 25°, pH 7.9 ± .1 and 0.1 M in sodium chloride unless otherwise noted.

b) Based on a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

c) Number of experiments performed for evaluation of K_o and k_o; second number refers to the number rejected by statistical reiterative evaluation procedure.

d) Reaction system 0.5 M in sodium chloride.

e) Reaction velocity less than 10^{-6} M min.$^{-1}$

C. DISCUSSION

An adequate and satisfying theory of the specificity of alpha-chymotrypsin-catalyzed reactions has only recently been developed (1a,b,c). In this theory, substrates represented by the formula $R_1CHR_2COR_3$, where $R_2 \neq H$, may combine with alpha-chymotrypsin through interaction of $R_1 = R_1'CONH$, R_2, COR_3 and H with their complementary loci, ρ_1, ρ_2 and ρ_3, and ρ_H present at the asymmetric active site of the enzyme. One cannot merely consider the interaction of each R group with its complementary ρ-locus, but must instead consider all possible permutations of $R_i - \rho_j$.* By use of the postulates developed in this theory, one can determine which interactions control the magnitude of the kinetic parameters k_o and K_o in equation 1. The theory has been extended to include bifunctional substrates, where $R_2 = H$ in the above formulation (2a,b).

For L-substrates the three interactions $R_i - \rho_i$* offer the optimal conditions for enzyme catalysis; however if one or more of the R groups is ineffective in this interaction, the compound may assume alternate orientations which are either non-productive or less reactive than the single optimum orientation. For the D-antipode, complete

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*In this thesis the notation $R_i - \rho_i$ will be used to signify the orientation involving the $R_1 - \rho_1$, $R_2 - \rho_2$, $R_3 - \rho_3$ in interactions. $R_i - \rho_i$ denotes the various permutations such as $R_1 - \rho_2$, $R_2 - \rho_3$, $R_1 - \rho_3$, $R_3 - \rho_2$, etc.

complementarity between the groups R_i, and the sites, ρ_1, including R_H - ρ_H, is not possible, and the alternate orientations determine both binding and reactivity.

If the two constants K_o and k_o are interpreted in terms of equations 2 and 3

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (2)$$

$$E + S \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES' \xrightarrow{k_4} E + P \qquad (3)$$

where $K_{s.1} = (k_{-1} + k_2)/k_1$ and $K_{s.2} = (k_{-3} + k_4)/k_3$, then

$$1/K_o = 1/K_{s.1} + 1/K_{s.2} \qquad (4)$$

or in the more general case of $\underline{n}$ binding modes

$$1/K_o = \sum_{i}^{n} 1/K_{s.i} \qquad (5)$$

It can be seen that values of K_o will vary between that of the lowest $K_{s.i}$ value when one binding mode is dominant to that of $K_{s.i}/n$ when each of the $\underline{n}$ binding modes leads to equivalent substration constants.

For the case where $k_4 = 0$, $\underline{i.e.}$, two binding modes, one productive and one non-productive,

$$k_o = K_{s.2}k_2/(K_{s.1} + K_{s.2}) \qquad (6)$$

and, generally,

$$k_o = k_2/(1 + K_{s.1} \sum_{j+1}^{n} 1/K_{s.j+1}) \qquad (7)$$

One can see that k_o will be decreased significantly whenever a non-productive binding mode competes successfully with a productive mode.

Attention is first called to the results in tables II, III and IV, in which kinetic constants for a series of acylated glycine, L-alanine, and D-alanine methyl esters are listed. The first two compounds in each table are for reference purposes. Since kinetic constants are unavailable for acetyl-D-alanine methyl ester, because of its very low order of reactivity, values have been estimated. In general, K_o for a D-compound is one-fourth to one-half the magnitude of that for the L-isomer, these values approaching the latter limit, one-half, when K_o is relatively large. Therefore, with an experimental value K_o = 611 mM for acetyl-L-alanine methyl ester, K_o = 300 mM was selected for the D-stereoisomer. Since this substrate was hydrolyzed too slowly to allow evaluation of the kinetic constants, and since values of k_o = 0.003 sec.$^{-1}$ have been measured, with some difficulty, for other substrates, $k_o \leq 0.001$ sec.$^{-1}$ was estimated for acetyl-D-alanine methyl ester.

A marked decrease in K_o for benzoylated aliphatic substrates relative to the corresponding acetylated substrates is expected (1a). This is due solely to the greater affinity

Table II

Kinetic Constants for a Series of Acylated Glycine Methyl Esters[a]

R_1 of $R_1NHCH_2CO_2CH_3$	K_o, mM	k_o, $sec.^{-1}$	Ref.
Acetyl	14	.0067	c, d, e
Benzoyl	5.3	.22	
Isonicotinoyl	18	.159	c, d, e
Nicotinoyl	31	.46	c, d, e
Picolinoyl	10	2.46	d, e
2-Furoyl	37	1.17	
2-Tetrahydrofuroyl	86	.143	
2-Thiophenoyl	3.1	.222	
o-Aminobenzoyl	2.4	.35	
2-Quinolinoyl	.35	.0067	

a) In aqueous solution at 25°, pH 7.90 ± .05 and 0.1 M in sodium chloride unless otherwise noted.

b) Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

c) Wolf, J.P., III, and Niemann, C., Biochem. 2, 82 (1963)

d) Reaction system 0.5 M in sodium chloride.

e) My own experiments have shown k_o to be ca. 20% smaller in 0.1 M sodium chloride than in 0.5 M sodium chloride. K_o is relatively unchanged over this range of sodium chloride concentrations.

Table III

Kinetic Constants for a Series of Acylated D-Alanine Methyl Esters[a]

R_1 of $R_1NHCH(CH_3)CO_2CH_3$	K_o, mM	k_o[b], $sec.^{-1}$	Ref.
Acetyl	(300)	(.001)	c
Benzoyl	2.4	.0073	d
Isonicotinoyl	27	.003	
Nicotinoyl	6	.0046	
Picolinoyl	17	.168	
2-Furoyl	19	.039	
2-Tetrahydrofuroyl	61	.017	
2-Thiophenoyl	7.4	.0159	
o-Aminobenzoyl	1.6	.005	
2-Quinolinoyl	Not a substrate		e

a) In aqueous solution at 25°, pH 7.90 ± .05 and 0.1 M in sodium chloride.

b) Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

c) Estimated constants, see text.

d) Unpublished experiments by W. A. Mukatis in these laboratories.

e) Reaction velocity less than 10^{-6} M $min.^{-1}$

Table IV

Kinetic Constants for a Series of Acylated L-Alanine Methyl Esters[a]

R_1 of $R_1NHCH(CH_3)CO_2CH_3$	K_o, mM	k_o^b, sec^{-1}	Ref.
Acetyl	611	1.29	c
Benzoyl	9.6	.23	d
Isonicotinoyl	29	.42	
Nicotinoyl	37	.58	
Picolinoyl	18	.071	
2-Furoyl	49	.51	
2-Tetrahydrofuroyl	132	.40	
2-Thiophenoyl	15	.46	
o-Aminobenzoyl	4.7	.24	
2-Quinolinoyl	.22	.004	

a) In aqueous solution at 25°, pH 7.90 ± .05 and 0.1 M in sodium chloride unless otherwise noted.

b) Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

c) Wolf, J.P., III, and Niemann, C., Biochem. 2, 493 (1963). Reaction solution 0.5 M in sodium chloride.

d) Unpublished experiments by W.A. Mukatis in these laboratories.

of the enzyme for an aromatic ring than for an aliphatic group. The magnitude of this decrease in K_o cannot be quantitatively predicted. Is the decrease from K_o = 611 mM for acetyl-L-alanine methyl ester to K_o = 9.6 mM for benzoyl-L-alanine methyl ester due solely to an increased R_2 - ρ_2 interaction in the productive enzyme-substrate complex? Or, does the above effect cause only part of the decrease in K_o, which is further lowered by the incidence of non-productive binding modes? Reference to Table IV and to the earlier algebraic arguments supports the latter alternative. Both K_o and k_o are abnormally low for benzoyl- and o-aminobenzoyl-L-alanine methyl ester. This is precisely the effect predicted by invoking competitive, non-productive modes of binding. Product is formed through the R_i - ρ_i complex. The kinetic parameters are altered by the R_3 - ρ_3, R_1 - ρ_2 orientation, which puts the methyl side chain into ρ_H*. Here the methyl side chain appears too small to be effective in orientation of the substrate into the R_i - ρ_i complex characterizing the usual trifunctional substrates of alpha-chymotrypsin. This apparent deficiency is further illustrated by the similarity of the kinetic constants for methyl hippurate to those for benzoyl-L-alanine methyl ester. In fact, the striking similarity between these two substrates

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*Reference 1c. p. 4498, Postulate 6: "Bulky groups occupying the ρH-locus lead to a dramatic decrease in values of k_o without necessarily causing a substantial change in corresponding values of K_o."

suggests that the methyl side chain exerts little influence on the alpha-chymotrypsin-catalyzed hydrolysis of benzoyl-L-alanine methyl ester. That the methyl side chain does have an influence, however, is shown by the differences between Tables II and IV, and by the observation of Hein and Niemann (1b) that inhibition of both benzoyl-D- and L-alanine methyl esters by indole is fully competitive.

The low K_o and higher k_o of benzoyl-D-alanine methyl ester relative to the constants of the acetylated compound are easily understood on the basis of predominant, productive binding with product being formed through the R_3 - ρ_3, R_1 - ρ_2 complex. Note that this is the same orientation dominant for the L-isomer where, however, it is non-productive. In this productive enzyme-substrate complex, the positioning of the carboalkoxy group is far less effective than that achieved in the case of L-substrates, as shown by the hundred-fold diminution in k_o. This poor positioning of the carbomethoxy group for the D-isomer is due to the lack of orienting alpha-acylamino group in the ρ_1 locus (1c).

The pattern for benzoylglycine methyl ester, relative to the acetyl analog, parallels that of the corresponding D-alanine methyl esters. Again, it appears that substitution of the aryl component for the methyl group increases productive binding. Note that in both the glycine and D-alanine, as in the L-alanine case, the benzoyl and o-aminobenzoyl

derivatives have unusually low K_o values relative to the rest of Tables II and III. A non-productive orientation is obviously exerting an influence on the observed substration constant.

If we neglect the quinolinoyl derivatives for the present and compare the kinetic constants in Tables II and III, a marked similarity is observed. In both tables, the acetyl derivative has the smallest k_o, the picolinoyl substrate the largest k_o and the furoylated compound the second largest k_o. In fact, the direction of change in the constants of any consecutive pair of substrates in one table is paralleled by the corresponding constants in the other table.

Turning our attention now to the kinetic constants for the acylated-L-alanine methyl esters, Table IV, and again neglecting momentarily quinolinoyl-L-alanine methyl ester, we see approximately the same variation of K_o as in Tables II and III. There is a much smaller variation in k_o and, in general, it is in the opposite direction from that in the previous tables. Whereas acetyl-D-alanine methyl ester and acetylglycine methyl ester have the lowest k_o of their series, acetyl-L-alanine methyl ester has the largest k_o of its series. Likewise, where the picolinoyl substrates in Tables II and III show the highest k_o values, picolinoyl-L-alanine methyl ester shows the lowest k_o value of the L-alanine methyl esters.

Another series of substrates showing similar properties to the L-alanine derivatives is shown in Table V, where the kinetic constants of a series of valine derivatives are listed. As in Table IV, the transition from acetyl to benzoyl involves a large decrease in both k_o and K_o. Also paralleling the L-alanine series, k_o for the picolinoyl substrate has the smallest value of the series.

These data suggest an explanation for the stereospecificity observed in alpha-chymotrypsin-catalyzed hydrolyses of trifunctional substrates. It has been argued previously that K_o for benzoyl-L-alanine methyl ester is significantly lowered by the non-productive R_1 - ρ_2 interaction, which largely determines K_o for the D-isomer where this interaction produces a productive orientation. It is to be expected, then, that any structural modification of a trifunctional substrate which would increase R_1 - ρ_2 interactions relative to the other R_1 interactions will decrease K_o for both optical antipodes. This effect increases the amount of non-productive binding by a trifunctional L-substrate thereby decreasing the value of k_o, while k_o will be increased for D-substrates where the R_1 - ρ_2 interaction gives a productive orientation. These changes are precisely those observed in Tables III and IV.

A test of this hypothesis is provided by Table VI, where a series of picolinoylated L-amino acid esters is compared to the corresponding series of acetylated L-amino

Table V

Kinetic Constants for a Series of Acylated L-Valine Methyl Esters[a]

R_1 of $R_1NHCH(CH(CH_3)_2)CO_2CH_3$	K_o mM	k_o^b $sec.^{-1}$	Ref.
Acetyl	112	.15	c
Benzoyl	4.6	.04	c
Nicotinoyl	5.7	.05	
Picolinoyl	10	.029	
2-Furoyl	19	.157	

a) In aqueous solution at 25°, pH 7.90 ± .05 and 0.1 M in sodium chloride.

b) Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

c) Waite, H.R., and Niemann, C., Biochem. 1, 250 (1962)

acid esters. In every example, k_o of the picolinoyl-substrate is less than that of the acetyl compound. In three of the cases, glycine, alanine, and valine, K_o for the benzoylated substrate is known, and as predicted, is substantially smaller than that for the picolinoylated substrate.

A final argument in favor of increased non-productive binding of acyl-L-alanine methyl esters relative to acetyl-L-alanine methyl ester is based upon the results of Table VII. If the lowering of k_o in the acylated-L-alanine methyl esters is due to increased wrong-way binding, one should be able to choose a suitably large side chain such that the acyl group would be unable to cause wrong-way, or non-productive, binding. This, indeed, is the case for both furoyl-L-phenylalanine methyl ester and tetrahydrofuroyl-L-phenylalanine methyl ester, where k_o is even slightly larger than k_o for the acetylated analog.

Tables II, III and IV suggest, also, a reason why bifunctional substrates have appeared qualitatively different from trifunctional compounds. Previously, glycine derivatives have always been compared to, and contrasted with, representative trifunctional substrates, i.e., acylated-L-amino acid esters. These tables show clearly the parallelism between this series of glycine substrates and the same series of acylated-D-alanine substrates. The tables show equally clearly the sharp contrast between the

TABLE VI

Kinetic Constants for a Series of Acylated L-Amino Acid Esters[a]

R_2 of $R_2CH(NHCOR_1')CO_2CH_3$	R_1' = Picolinoyl		R_1' = Acetyl	
	K_o mM	k_o sec.$^{-1}$	K_o mM	k_o^b sec.$^{-1}$
H	11.6	2.02	31	.013[c,d]
CH_3	18	.071	611	1.29[c,d]
CH_3CH_2	9	.36	54	1.05[e]
$CH_3CH_2CH_2$	5	1.9	10	2.7[f]
$(CH_3)_2CH$	10	.029	112	.15[g]

a) In aqueous solution at 25°, pH 7.90 ± .05 and 0.1 M in sodium chloride unless otherwise noted.

b) Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

c) Wolf, J.P., III, and Niemann, C., Biochem. 2, 82 (1963).

d) Reaction system 0.5 M in sodium chloride.

e) Unpublished experiments of Dr. J.B. Jones in these laboratories.

f) Jones, J.B., and Niemann, C., Biochem. 1, 1093 (1962).

g) Waite, H.R., and Niemann, C., Biochem. 1, 250 (1962).

Table VII

Kinetic Constants of Three Acylated L-Phenylalanine Esters[a]

R_1 of $R_1NHCH(CH_2C_6H_5)CO_2CH_3$	K_o mM	k_o^b sec.$^{-1}$	Ref.
Acetyl	1.25	53	c
2-Furoyl	0.36	74	
2-Tetrahydrofuroyl	1.2	95	

a) In aqueous solution at 25°, pH 7.90 and 0.1 M in sodium chloride.

b) Based on a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

c) Jones, J.B. and Niemann, C., Biochem. 2, 498 (1963).

glycine derivatives and the trifunctional-L-substrates. The conclusion is drawn, with some confidence, that the enzyme-catalyzed hydrolysis of this series of bifunctional substrates proceeds through the R_1 - ρ_2, R_2 - ρ_1 and R_3 - ρ_3 orientation just as does the hydrolysis of D-substrates.

Because of the two alpha protons of glycine there are two orientations allowing both R_3 - ρ_3 and H - ρ_H interactions. One of these involves an R_1 - ρ_1 interaction, similar to an L-substrate, while the other involves an R_1 - ρ_2 interaction, similar to the D-substrate. It seems reasonable that suitable variation of the acyl moiety could favor either orientation predominantly or even a combination of the two. One might study the enzyme-catalyzed hydrolysis of the methyl esters of glycine, L-alanine and D-alanine as the $R_1'CO$ group is varied through a homologous series of aliphatic acyl groups, such as acetyl, propionyl, etc., to see whether the bifunctional series parallels more closely the D- or L-trifunctional series.

Reference to equation 1 shows that at very low substrate concentration a second-order rate expression is obeyed. An apparent second-order rate constant can be extracted which is equal to k_o/K_o. This ratio offers a numerical evaluation of a compound's effectiveness as a substrate. In fact the stereospecificity of alpha-chymotrypsin for a given pair of antipodes can be quantitated by comparing the apparent bimolecular rate constants for the two

isomers, $(k_o/K_o)_L/(k_o/K_o)_D$. These ratios have been tabulated in Table VIII. The 1600-fold variation in stereospecificity shows that the nature of the group R_1' in compounds of the type $R_1'CONHCHR_2COR_3$, with R_2 and R_3 remaining invariant, can determine the degree of stereochemical preference for a given antipode or even cause an inversion of the antipodal specificity usually observed for alpha-chymotrypsin-catalyzed reactions, i.e., preference for the L-antipode.

It is apparent that positioning of the hetero-atom in the acyl moiety has a great influence on the degree of stereospecificity of the substrates containing a hetero-atom in the acyl group. Isonicotinoylalanine methyl ester, where the nitrogen atom is para to the carbonyl group, shows the highest degree of stereospecificity. When the hetero-atom is moved to the meta position, as in nicotinoylalanine methyl ester, the stereospecificity is decreased by 6-fold. When the hetero-atom is now moved to the ortho-position the observed degree of stereospecificity is the lowest ever found for alpha-chymotrypsin-catalyzed reactions and in fact, favors the D-antipode.

The effect of the hetero-atom in the acyl group on the value of k_o in Table III is very instructive. Clearly, nitrogen, oxygen and sulfur cause a significant increase in k_o of the acylated-D-alanine methyl ester. The effect decreases in the order nitrogen > oxygen > sulfur. This may

Table VIII

The Effect of the Acyl Moiety on
the Degree of Stereospecificity

R_1CO of $R_1CONHCH(CH_3)CO_2CH_3$	$(k_o/K_o)_L$	$(k_o/K_o)_D$	$(k_o/K_o)_L(k_o/K_o)_D^{-1}$
Acetyl	2.1	.0033	635
Benzoyl	24	3	8
Isonicotinoyl	14	.11	127
Nicotinoyl	16	.8	20
Picolinoyl	3.9	9.9	0.39
Furoyl	10	2	5
Tetrahydrofuroyl	3	.28	11
2-Thiophenoyl	31	2.1	15
o-Aminobenzoyl	51	3.1	16

be a function of either the basicity or nucleophilicity of the hetero-atom involved.

The usual mechanisms proposed for alpha-chymotrypsin catalysis entail either general basic or nucleophilic catalysis by an imidazole nitrogen present at the active site (7). It is interesting to speculate that a suitably placed general base or nucleophile on a substrate molecule might assist or even supersede that hetero-atom already present as a part of the enzyme. One type of evidence supporting the participation of an imidazole nitrogen atom in alpha-chymotrypsin catalysis is the pH-activity curve suggesting a group having the pK of imidazole (8-10). If, then, the above hypothesis is true, one would expect quite a different pH-activity profile for the substrates having effective hetero-atoms in the acylamido group. Assessment of the validity of this speculation will have to await the time when a rough spatial structure of the enzyme-substrate complex is known.

The quinolinoylated compounds were excluded from the preceding discussion, not because they are not subject to the same effects as the other substrates examined, but because they possess structural modifications which override the above effects. The uniformly low k_o values of these substrates are close to the limits of measurement in the glycine and L-alanine cases and immeasurable in the case of D-alanine. They provide an example of a substrate in

which one group is so strongly attracted by the enzyme, that all other factors including orientation of the carboalkoxy group are overridden, and while a strong substrate-enzyme interaction is achieved (more than ten times greater than with the other substrates discussed), substrate activity is very nearly lost. This orientation factor need not be the only one operating to diminish k_o for the quinolinoyl derivatives. There is obviously structural specificity within each ρ-locus. For example, O-alkylation of N-acetyl-L-tyrosine methyl ester can reduce k_o by a factor of 10^4 (11). The same factor, presumably steric, operating to lessen k_o of the tyrosine derivatives may be operative in the quinolinoyl derivatives.

Since the observed degree of stereospecificity is very high in acylated aromatic amino acid esters and relatively low in the corresponding alanine derivatives, one might expect other aliphatic amino acid esters to show an intermediate degree of stereospecificity. However, even an intermediate degree of stereospecificity would mean such low values for k_o for the D-antipodes that these might easily go undetected. Since the highest k_o for the D-alanine derivatives was found with a picolinoyl acyl group, this is the acyl group to use when looking for reactivity of other D-amino acid esters. On the basis of these arguments, an attempt was made to observe enzyme-catalyzed hydrolysis of picolinoyl-D-valine methyl ester. No such catalysis was

observed. If this compound were to be hydrolyzed via the same productive orientation as picolinoyl-D-alanine methyl ester, it would require an isopropyl- ρ_1 interaction. Such an interaction may be sterically similar to that of an N-methyl acetamido- ρ_1 interaction which has been shown to cause a 10^4-fold decrease in k_o relative to the non-methylated acetamido compound (11). Such a decrease in k_o of picolinoyl-D-valine methyl ester would render it experimentally inactive as a substrate. An attempt should be made to observe alpha-chymotrypsin-catalyzed hydrolysis of D-alpha-picolinamido methyl-n-butyrate, where the above steric effect would not be operative.

27 to 30

D. PUBLICATION

The pages indicated above contain the text of an article which was published in the Journal of the American Chemical Society 85, 1896 (1963):

"A Further Example of Inversion of the Usual Antipodal Specificity of Alpha-Chymotrypsin"*

by James R. Rapp and Carl Niemann

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*Contribution No. 2948 from the Gates and Crellin Laboratories of Chemistry

Sir:

In 1948 it was shown that the stereochemical course of the papain catalyzed synthesis of alpha-N-acylated alpha-amino acid phenylhydrazides, from certain alpha-N-acylated amino acids and phenylhydrazine, could be determined by the structure of the acyl component (12). Subsequent studies (13-15) confirmed and extended these results but attempts to observe the same phenomenon with the comparable alpha-chymotrypsin catalyzed reaction were unsuccessful (16).

In 1960 an inversion of the usual antipodal specificity of alpha-chymotrypsin was demonstrated when it was found that the rate of alpha-catalyzed hydrolysis of D-3-carbomethoxy-dihydroisocarbostyril, to the corresponding acid, was markedly greater than that of the L-antipode (17). In providing an explanation for the preceding observations a theory was developed (1a,b,c) which not only accounted for the above results but also forecast in general terms the existence of other examples of inversion of antipodal specificity as well as those involving diminished stereochemical preference in favor of the L-antipode for compounds of the type $R_1'CONHCHR_2COR_3$ and cognate structures.

In a recent communication Cohen, et al. (18), describe an inversion of the usual antipodal specificity in the alpha-chymotrypsin catalyzed hydrolysis of ethyl alpha-acetoxypropionate. This behavior was explained in terms of theory (18), which was similar to that developed earlier

(1a,b,c), and provided a needed example of inversion of antipodal specificity in a case where the structures were not conformationally constrained. However, there remained a need for a demonstration that the nature of the group R_1' in compounds of the type $R_1'CONHCHR_2COR_3$, with the nature of R_2 and R_3 remaining invariant, could determine the degree of stereochemical preference for a given antipode or cause an inversion of the antipodal specificity usually observed for alpha-chymotrypsin catalyzed reactions, i.e., preference for the L-antipode.

An example of diminished stereochemical preference for the L-antipode in compounds of the type $R_1'CONHCHR_2COR_3$, and associated with the nature of R_1', became available when it was found that the kinetic constants for the alpha-chymotrypsin catalyzed hydrolysis of N-benzoylalanine methyl ester, in aqueous solutions at 25.0°, pH 7.90 and 0.20M in sodium chloride, were K_o = 3.3 ± 0.2mM and k_o = 0.0107 ± 0.0002 $sec.^{-1}$ for the D-antipode, and K_o = 9.8 ± 0.9 mM and k_o = 0.26 ± 0.01 $sec.^{-1}$ for the L-antipode (1b,c). For N-acetylalanine methyl ester, K_o = 611 ± 10 mM and k_o = 1.29 ± 0.02 $sec.^{-1}$ for the L-antipode (in 0.50 M sodium chloride) with no detectable substrate activity being observable for the D-antipode (2b).

The sought for example of inversion of antipodal specificity for substrates of the type $R_1'CONHCHR_2COR_3$ arising from appropriate selection of the group R_1', with the nature of R_2 and R_3 remaining invariant, has now been

found. In the alpha-chymotrypsin catalyzed hydrolysis of N-picolinylalanine methyl ester, in aqueous solutions at 25.0°, pH 7.90 and 0.10 M in sodium chloride, values of K_o = 18 ±1mM and k_o = 0.070 ± 0.003 sec^{-1} were obtained for the L-antipode (m.p. 59-60°, $(\alpha)_D^{25}$ -15.3 ± 0.3° (c. 3% in water)) and K_o = 17 ± 1 mM and k_o = 0.165 ± 0.006 sec^{-1} for the D-antipode (m.p. 59-60°, $(\alpha)_D^{25}$ 15.3 ± 0.3° (c. 3% in water)). The experiments were conducted with the aid of a pH-stat (3,19) under conditions where (E) = 26 μM) and (S) = 2.3-18.4 mM for the L-antipode and (E) = 74 μM and (S) = 1.5-12 mM for the D-antipode. The primary data were evaluated using a Datatron 220 digital computer programmed as described previously (6).

With three examples of inversion of the usual antipodal specificity of alpha-chymotrypsin involving both conformationally constrained and unconstrained substrates, two of which are alpha-N-acylated alpha-amino acid derivatives, it is evident that substantial support has now been provided for the explanation of this phenomenon given earlier (1a,b,c). It also follows that the more general theory (1a,b,c) which envisions non-productive combination of substrate that is fully competitive with its productive combination with the active site of the enzyme has acquired added significance.

This research was supported in part by a grant from the National Institutes of Health, U.S. Public Health Service.

E. EXPERIMENTAL*

(i) alpha-Chymotrypsin

Two preparations of alpha-chymotrypsin were used in these studies, Armour salt free, bovine, lot #T97207 and Worthington C.D.I. 6066-67. Nitrogen determinations on these and a third preparation, not used herein, were performed by the micro-Kjeldahl procedure of Redemann (20).**

Preparation	% Protein Nitrogen
Armour T97207	13.97 ± .11
Worthington C.D.I. 6066-67	14.91 ± .06
Worthington C.D.C. 46	13.17 ± .08

(ii) Methyl Picolinurate

This compound was synthesized by a mixed anhydride method (21). To a cooled solution of isobutylchlorocarbonate in chloroform was added a chloroform solution containing one equivalent of picolinic acid hydrochloride and two equivalents triethyl amine. This solution was kept below 0° for 20-30 minutes after which time was added a chloroform solution containing one equivalent glycine methyl ester hydrochloride and one equivalent of triethyl

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*All melting points corrected by a calibration curve of Dr. A. Platt. All optical rotations were measured at room temperature, 25 ± 2° C. and have an uncertainty of ± 2-3%. Analyses are by Spang Microanalytical Laboratory, Ann Arbor, Michigan.

**I am indebted to Mr. G. Neil, who shared the labor of these analyses.

amine. After 12 hours, the solution was washed once with 1 N HCl and twice with saturated aqueous $NaHCO_3$, dried over Na_2SO_4 and the solvent removed under reduced pressure. The remaining oil was distilled at 136° and 380 μ Hg. Lit. (22) b.p. 123° and 200 μ Hg. The distillate crystallized readily and was recrystallized from diisopropyl ether to give fine needles, m.p. 47-48°.

Analysis:	calculated	C: 55.7	H: 5.2	N: 14.4
$C_9H_{10}N_2O_3$(194)	found	C: 55.9	H: 5.3	N: 14.4

(iii) Methyl-2-quinolinurate

This was prepared by a mixed anhydride synthesis on 2-quinolinic acid as in ii. Four recrystallizations from diisopropyl ether gave white plates, m.p. 100.5-101.5°.

Analysis:	calculated	C: 63.9	H: 5.0	N: 11.5
$C_{13}H_{12}N_2O_3$(244)	found	C: 64.1	H: 5.3	N: 12.2

(iv) 2-Thiophenoylglycine methyl ester

This was prepared by a mixed anhydride synthesis on 2-thiophenic acid as in ii. Three recrystallizations from diisopropyl ether gave long, silky threads, m.p. 93-94°.

Analysis:	calculated	C: 48.2	H: 4.6	N: 7.0	S: 16.1
$C_8H_9NO_3S$(199)	found	C: 48.1	H: 4.6	N: 6.8	S: 16.3

(v) o-Amino methyl hippurate

To equivalent amounts of isatoic anhydride and glycine methyl ester hydrochloride was added a chloroform solution containing one equivalent triethyl amine. The mixture was refluxed until evolution of CO_2 ceased, ca. two hours. The reaction mixture was washed with saturated aqueous $NaHCO_3$, dried over Na_2SO_4 and the chloroform removed under reduced pressure to leave a dark oil. The oil was taken up in methanol, treated with Norit, diluted with an equal volume diisopropyl ether and refrigerated. The resulting crystals were recrystallized three times from a mixture of methanol and diisopropyl ether to give colorless needles, m.p. 90.5-91.5°.

Analysis:	calculated	C: 57.7	H: 5.8	N: 13.5
$C_{10}H_{12}N_2O_3$(208)	found	C: 57.8	H: 5.8	N: 13.0

(vi) Tetrahydrofuroic acid

2-Furoic acid (50 g.), glacial acetic acid (200 ml.), and Pd-C (2 g.) catalyst were put under 50 psi. H_2 for 24 hours. The acetic acid was distilled at atmospheric pressure followed by distillation of tetrahydrofuroic acid at 145-150° and 25-30 mm.Hg. Lit. (23) b.p. 131-132° and 14 mm.Hg.

(vii) Methyl tetrahydrofurourate

This was prepared by carrying out a mixed anhydride

synthesis on tetrahydrofuroic acid as in ii. Two distillations at 92-93° and 90 μ Hg gave a clear liquid, $(n)_D^{25}$ 1.4735, showing the expected infra-red spectrum.

Analysis:	calculated	C: 51.3	H: 7.0	N: 7.5
$C_8H_{13}NO_4$(187)	found	C: 51.2	h: 7.1	N: 7.3

(viii) Isonicotinoyl-L-alanine methyl ester

This was prepared by a mixed anhydride synthesis on isonicotinic acid as in ii. To the resulting anhydride was added one equivalent L-alanine methyl ester hydrochloride in chloroform containing one equivalent triethyl amine. Two recrystallizations from methanol - diisopropyl ether gave colorless prisms, m.p. 85.5-86.5°. $(\alpha)_D$ -29.4° (c. 5% in H_2O).

Analysis:	calculated	C: 57.7	H: 5.8	N: 13.5
$C_{10}H_{12}N_2O_3$(208)	found	C: 57.8	H: 5.9	N: 13.5

(ix) Isonicotinoyl-D-alanine methyl ester

This was prepared as the above compound, viii, with the substitution of D-alanine methyl ester hydrochloride for the L-isomer. Two recrystallizations from methanol - diisopropyl ether gave colorless prisms, m.p. 85.5-86.5°. $(\alpha)_D$ 29.6° (c. 5% in H_2O).

Analysis:	calculated	C: 57.7	H: 5.8	N: 13.5
$C_{10}H_{12}N_2O_3$(208)	found	C: 57.8	H: 5.9	N: 13.4

(x) Nicotinoyl-L-alanine methyl ester

This was prepared from a mixed anhydride synthesis with nicotinic acid. The product, after three recrystallizations from diisopropyl ether, consisted of long, colorless needles, m.p. 75.5-76.5°. $(\alpha)_D$ -30.6° (c. 5% in H_2O).

Analysis:	calculated	C: 57.7	H: 5.8	N: 13.5
$C_{10}H_{12}N_2O_3$(208)	found	C: 57.1	H: 6.0	N: 13.6

(xi) Nicotinoyl-D-alanine methyl ester

This was prepared as above. Three recrystallizations from diisopropyl ether gave colorless needles, m.p. 75.5-76.5°. $(\alpha)_D$ 30.0° (c. 5% in H_2O).

Analysis:	calculated	C: 57.7	H: 5.8	N: 13.5
$C_{10}H_{12}N_2O_3$(208)	found	C: 57.5	H: 6.0	N: 13.6

(xii) Picolinoyl-L-alanine methyl ester

This was prepared through a mixed anhydride synthesis using picolinic acid hydrochloride. After three recrystallizations, colorless plates resulted, m.p. 59-60°. $(\alpha)_D$ -15.3° (c. 3% in H_2O).

Analysis:	calculated	C: 57.7	H: 5.8	N: 13.5
$C_{10}H_{12}N_2O_3$(208)	found	C: 57.6	H: 6.1	N: 13.4

(xiii) Picolinoyl-L-alanine methyl ester

This was prepared as the above L-isomer. Three recrystallizations from diisopropyl ether gave colorless plates, m.p. 59-60°. $(\alpha)_D$ 15.3° (c. 3% in H_2O).

Analysis:	calculated	C: 57.7	H: 5.8	N: 13.5
$C_{10}H_{12}N_2O_3$(208)	found	C: 57.8	H: 5.9	N: 13.3

(xiv) Furoyl-L-alanine methyl ester

To a cooled solution of L-alanine methyl ester hydrochloride in chloroform was added one equivalent triethyl amine. 2-Furoyl chloride (one equivalent) was taken up in chloroform and added dropwise to the above cooled solution. After 24 hours the reaction mixture was washed with 1 **N** hydrochloric acid and with saturated aqueous $NaHCO_3$. The chloroform layer was dried over Na_2SO_4 and evaporated. The residual solid was recrystallized three times from diisopropyl ether giving colorless crystals, m.p. 41-42°. $(\alpha)_D$ -2.8° (c. 6% in H_2O).

Analysis:	calculated	C: 54.8	H: 5.6	N: 7.1
$C_9H_{11}NO_2$(197)	found	C: 54.6	H: 5.9	N: 7.3

(xv) Furoyl-D-alanine methyl ester

This was prepared in the same manner as the above isomer. Three crystallizations from diisopropyl ether gave colorless crystals, m.p. 41-42°. $(\alpha)_D$ 2.7° (c. 6% in H_2O).

Analysis:	calculated	C: 54.8	H: 5.6	N: 7.1
$C_9H_{11}NO_4$(197)	found	C: 54.9	H: 5.9	N: 7.2

(xvi) Tetrahydrofuroyl-L-alanine methyl ester

This was prepared in the same way as the glycine analog, vii, with L-alanine methyl ester hydrochloride used in place of the glycine ester hydrochloride. Two distillations at 86-88° and 200 μHg gave a clear liquid. $(n)_D^{25}$ 1.4671. $(\alpha)_D$ -29.3° (c. 6% in methanol).

Analysis:	calculated	C: 53.7	H: 7.5	N: 6.9
$C_9H_{15}NO_4$(201)	found	C: 53.6	H: 7.3	N: 6.7

(xvii) Tetrahydrofuroyl-D-alanine methyl ester

This was prepared through a mixed anhydride synthesis using the same procedure as on the L isomer above. Two distillations at 95-96° and 300 μ Hg gave a clear liquid, $(n)_D^{25}$ 1.4675. $(\alpha)_D$ 29.2° (c. 6% in methanol)

Analysis:	calculated	C: 53.7	H: 7.5	N: 6.9
$C_9H_{15}NO_4$(201)	found	C: 53.8	H: 7.6	N: 6.9

(xviii) 2-Thiophenoyl-L-alanine methyl ester

The synthesis of this compound proceeded smoothly through the addition of L-alanine methyl ester to a mixed anhydride of 2-thiophenic acid and isobutylchlorocarbonate. Two recrystallizations from methanol - diisopropyl ether gave colorless prisms, m.p. 91-92°. $(\alpha)_D$ -16.0° (c. 4%

in methanol).

Analysis:	calculated	C: 50.7	H: 5.2	N: 6.6	S: 15.0
$C_9H_{11}NO_3S$(213)	found	C: 50.8	H: 5.2	N: 6.6	S: 14.9

(xix) 2-Thiophenoyl-D-alanine methyl ester

This was prepared through the same anhydride used in the preparation of the L-isomer. After two recrystallizations from methanol - diisopropyl ether, the colorless prisms exhibited m.p. 91-92°. $(\alpha)_D$ 15.5° (c. 4% in methanol).

Analysis:	calculated	C: 50.7	H: 5.2	N: 6.6	S: 15.0
$C_9H_{11}NO_3S$(213)	found	C: 50.8	H: 5.2	N: 6.3	S: 15.0

(xx) o-Aminobenzoyl-L-alanine methyl ester

This was prepared by the refluxing of a chloroform solution containing one equivalent each of isatoic anhydride, L-alanine methyl ester hydrochloride and triethyl amine. Three recrystallizations from methanol - diisopropyl ether gave colorless needles, m.p. 92.5-93.5°. $(\alpha)_D$ -70.0° (c. 4% in methanol).

Analysis:	calculated	C: 59.5	H: 6.3	N: 12.6
$C_{11}H_{14}N_2O_3$(222)	found	C: 59.5	H: 6.3	N: 12.7

(xxi) o-Aminobenzoyl-D-alanine methyl ester

This was prepared in the same way as the L-isomer, xx. Four recrystallizations from methanol - diisopropyl ether

gave colorless needles, m.p. 92.5-93.5°. $(\alpha)_D$ 70.0° (c. 4% in methanol).

Analysis:	calculated	C: 59.5	H: 6.3	N: 12.6
$C_{11}H_{14}N_2O_3$(222)	found	C: 59.3	H: 6.2	N: 12.6

(xxii) 2-Quinolinoyl-L-alanine methyl ester

This was prepared by addition of a chloroform solution of L-alanine methyl ester hydrochloride and triethyl amine to a cooled preparation of the mixed anhydride from isobutylchlorocarbonate and 2-quinolinic acid. After three recrystallizations from methanol - diisopropyl ether the long silky threads melted at 60.5-61.5°. $(\alpha)_D$ 83.0° (c. 4% in chloroform).

Analysis:	calculated	C: 65.1	H: 5.5	N: 10.8
$C_{14}H_{14}N_2O_3$(258)	found	C: 65.1	H: 5.5	N: 10.7

(xxiii) 2-Quinolinoyl-D-alanine methyl ester

This was prepared in the same way as the L-isomer, xxii. Two recrystallizations gave long, silky threads, m.p. 60.5-61.5°. $(\alpha)_D$ -83.3° (c. 4% in chloroform).

Analysis:	calculated	C: 65.1	H: 5.5	N: 10.8
$C_{14}H_{14}N_2O_3$(258)	found	C: 64.7	H: 5.4	N: 10.8

(xxiv) Nicotinoyl-L-valine methyl ester

This was prepared by the same procedure used to synthesize nicotinoyl-L-alanine methyl ester, x. Two

recrystallizations from diisopropyl ether gave colorless needles, m.p. 102-103°. $(\alpha)_D$ 38.8° (c. 4% in chloroform).

Analysis:	Calculated	C: 61.0	H: 6.8	N: 11.9
$C_{12}H_{16}N_2O_3$(236)	found	C: 60.7	H: 6.8	N: 12.2

(xxv) Picolinoyl-L-valine methyl ester

This was synthesized through the mixed anhydride of picolinic acid and isobutylchlorocarbonate in the same manner as picolinoyl-L-alanine methyl ester, xii, was prepared. Three distillations at 130-135° and 75 μHg gave a colorless viscous liquid. $(n)_D^{25}$ 1.5090. $(\alpha)_D$ 31.8° (c. 4% in chloroform).

Analysis:	calculated	C: 61.0	H: 6.8	N: 11.9
$C_{12}H_{16}N_2O_3$(236)	found	C: 60.9	H: 6.9	N: 12.1

(xxvi) Picolinoyl-D-valine methyl ester

This synthesis was carried out just as was that of the L-isomer, xxv. Three distillations at 125-130° and 70 μHg gave a clear, colorless liquid. $(n)_D^{25}$ 1.5086. (α) -31.0° (c. 4% in chloroform).

Analysis:	calculated	C: 61.0	H: 6.8	N: 11.9
$C_{12}H_{16}N_2O_3$(236)	found	C: 61.0	H: 6.9	N: 12.0

(xxvii) Furoyl-L-valine methyl ester

Synthesis of this compound was carried out in the same way as that of furoyl-L-alanine methyl ester, xiv. Two

recrystallizations from diisopropyl ether gave long, silky threads, m.p. 80-81°. $(\alpha)_D$ 41.3° (c. 4% in chloroform).

Analysis:	calculated	C: 58.7	H: 6.7	N: 6.2
$C_{11}H_{15}NO_4$(225)	found	C: 58.7	H: 6.6	N: 6.3

(xxviii) L-alpha-Picolinamido methyl-n-butyrate

L-alpha-Amino-n-butyric acid was esterified by the method of Brenner and Huber (24) to give L-alpha-amino methyl-n-butyrate hydrochloride. This compound was acylated by the mixed anhydride procedure used in ii. Three distillations at 124-125° and 200 μ Hg gave a pale yellow liquid, $(n)_D^{25}$ 1.5155. $(\alpha)_D$ 27.2° (c. 5% in chloroform).

Analysis:	calculated	C: 59.4	H: 6.3	N: 12.6
$C_{11}H_{14}N_2O_3$(222)	found	C: 59.3	H: 6.2	N: 12.7

(xxix) Picolinoyl-L-norvaline methyl ester

This was synthesized by the same procedure as compound xxviii. Three distillations at 113-115° and 75 μ Hg gave a pale yellow liquid. $(n)_D^{25}$ 1.5090. $(\alpha)_D$ 22.8° (c. 4% in chloroform).

Analysis:	calculated	C: 61.0	H: 6.8	N: 11.9
$C_{12}H_{16}N_2O_3$	found	C: 60.9	H: 6.9	N: 11.7

(xxx) Furoyl-L-phenylalanine methyl ester

L-phenyl alanine was esterified by the method of Brenner and Huber (23). The methyl ester hydrochloride thus

obtained was acylated with furoyl chloride as in xiv. Three recrystallications from methanol - diisopropyl ether gave colorless needles, m.p. 73-74°. $(\alpha)_D$ -60.4° (c. 5% in methanol).

Analysis:	calculated	C: 65.9	H: 5.5	N: 5.1
$C_{15}H_{15}NO_4$(273)	found	C: 65.9	H: 5.4	N: 5.1

(xxxi) Tetrahydrofuroyl-L-phenylalanine methyl ester

This was prepared from the mixed anhydride between isobutylchlorocarbonate and tetrahydrofuroic acid as in vii. Two distillations at 160-161° and 100 μ Hg gave a pale yellow, viscous oil. $(n)_D^{25}$ 1.5240. $(\alpha)_D$ 46.0° (c. 5% in chloroform).

Analysis:	calculated	C: 64.9	H: 6.9	N: 5.0
$C_{15}H_{19}NO_4$(277)	found	C: 64.8	H: 6.9	N: 5.0

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PART II

THE STRUCTURAL SPECIFICITY OF ALPHA-CHYMOTRYPSIN:

SOME NEW SUBSTRATES

A. INTRODUCTION

This section is composed of two parts which, to a large extent, are unrelated. They are considered together for the sake of convenience.

In the first part, the effect on the kinetic constants of reducing an aromatic substrate to its hexahydroaromatic counterpart is considered. Two classes of substrates are distinguished, one in which the ring is in the side chain and the other where the ring is part of the acylamido group.

In the second part, several analogs of known substrates are examined and their relationship and importance to the general picture of the specificity of alpha-chymotrypsin are discussed.

B. RESULTS

The results are summarized in Table IX*.

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*For a description of the kinetic procedure used, see Part I of this thesis.

Table IX

The α-Chymotrypsin-Catalyzed Hydrolysis of a Variety of New Substrates[a]

Substrate	$(S)_o$, mM	$(E)_o$, M[b]	No. of[c] Expts.	K_o, mM	k_o, $sec.^{-1}$
N-Acetyl-L-phenylglycine methyl ester	1.2 -9.9	3.4	14-0	2.4±.3	.88 ± .03
N-Acetyl-L-H_6-phenylglycine methyl ester	.51-10	3.6	14-0	1.3±.1	.264± .005
Hexahydro methyl hippurate	4.3 -34	34	12-2	34 ± 3	.067± .004
Hexahydrobenzoyl-L-alanine methyl ester	1.9 -69	32	18-2	58 ± 8	.55 ± .06
Hexahydrobenzoyl-D-alanine methyl ester	20	65	Not a substrate[d]		
Hexahydrobenzoyl-L-valine methyl ester	.18-.73	62	Insoluble above first order range $K_o > 10$		k_α
B-Benzoyl methyl propionate[e]	.11-.89	6.8	16-0	.29±.08	.19 ± .02
Benzoyl acetic acid methyl ester	.43-3.5	38	8-0	1.01±.08	.047±.001
N-(p-hydroxyphenyl)-glycine methyl ester	.23-1.9	48	10-0	2.5 ± .3	.099±.006
N-phenyl-dl-alanine methyl ester[e]	.78-6.3	33	15-0	1.8 ± .8	.0035±.0005

a) In aqueous solution at 25°, pH 7.9 ± .1 and 0.1 M in sodium chloride unless otherwise noted.

b) Based on a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

c) Number of experiments performed for evaluation of K_o and k_o; second number refers to the number rejected by statistical reiterative evaluation procedure.

d) Reaction velocity less than 10^{-6} M $min.^{-1}$.

e) Reaction system 0.02 M in sodium chloride.

C. DISCUSSION

1. Aromatic-Hexahydroaromatic Substrates

It has been stated repeatedly that the affinity of α -chymotrypsin is greater for aromatic substrates than for aliphatic substrates. This affinity of the active site of the enzyme for aromatic compounds has been attributed to bulk (1), to a specific attraction for the aromatic π-electron system (2), and to the planarity of the aromatic group which gives an assumed greater effective area of contact in its interaction with the active site (3). One method of distinguishing between the first type of enzyme-aromatic interaction and the latter two types is to study the α-chymotrypsin-catalyzed hydrolysis of analogous aromatic and alicyclic substrates. If bulk is the major factor, reduction of the aromatic ring should have little effect, whereas if the π- electron system or planarity is important, reduction will have a very great effect on the kinetic constants. The only examples of this are the two pairs listed in Table X. Some supplementary data on inhibition studies are shown in Table XI. Table XI gives a straightforward prediction of a several-fold preference of alpha-chymotrypsin for aromatic compounds. The opposite appears to be true of the substrates in Table X.

The initial intent was to study several pairs of aromatic-hexahydroaromatic amino acid derivatives. At the time, the paucity of aromatic amino acids was realized but

Table X

Kinetic Constants of Aromatic-Hexahydroaromatic Pairs of Substrates[a]

Substrate	K_o mM	k_o^b $sec.^{-1}$	Ref.
Acetyl-L-phenylalaninamide	34	.048	(1)
Acetyl-L-H_6phenylalaninamide	27	.041	(1)
Acetyl-L-phenylalanine methyl ester	1.2	52	(4)
Acetyl-L-H_6phenylalanine methyl ester	.18	13	(4)

a) In aqueous solution at 25° and pH 7.90 ± .05

b) Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

Table XI

Inhibition Constants for Some Aromatic-Hexahydroaromatic Inhibitors

Inhibitor	K_I mM	Ref.
Benzene	25	a
Cyclohexane	70	
Phenol	6	b
Cyclohexanol	75	
Phenyl acetate	42	(2)
Cyclohexyl acetate	86	(2)
β -Phenyl propionate	5.5	(2)
β -Cyclohexyl propionate	30	(2)
γ -Phenyl butyrate	14	(2)
γ -Cyclohexyl butyrate	35	(2)

a) Part III of this thesis.

b) Wallace, R.A., Kurtz, A.N. and Niemann, C., Biochem. 2, 824 (1963)

the added difficulty due to insolubility of hexahydro-aromatic compounds was not anticipated. Unfortunately, only one aromatic-alicyclic pair with the ring on the side chain was studied. This consists of N-acetyl-L-phenylglycine methyl ester and its reduced derivative, N-acetyl-L-hexahydrophenylglycine methyl ester (the first two compounds in Table IX). Here, as in the case of the phenylalanine-hexahydrophenylalanine methyl ester, both K_o and k_o were lower for the alicyclic compound. These data, limited as they are, support the hypothesis of Jones and Niemann (4) that the K_o is depressed by an energetically more favorable interaction of the enzyme-hexahydrosubstrate, while the lowering of k_o is due to the deformability of the cyclo-hexyl group which is itself deformed upon combination with the ρ_2 locus rather than effecting alteration of the conformation of the site.

In an attempt to circumvent the problem of too few aromatic amino acids, attention was turned to pairs of substrates possessing an aromatic or alicyclic acylamido group. Here, the aromatic or aliphatic ring is not present in the side chain but in the alpha-acylamido moiety. These compounds are shown in Table XII in order of increasing size of side chain, i.e., glycine, alanine, valine and phenylalanine. The results for these compounds are quite different from those where the ring was in the side chain. In every case, K_o is several-fold greater for the hydroaromatic substrate than for the aromatic amino acid ester. The changes

TABLE XII

Kinetic Constants of Aromatic-Hexahydroaromatic Pairs of Substrates[a]

Substrate	K_o, mM	k_o^b, sec.$^{-1}$	Ref.
Methyl hippurate	5.3	.22	
Methyl hexahydrohippurate	34	.067	
Benzoyl-L-alanine methyl ester	9.6	.23	c
Hexahydrobenzoyl-L-alanine methyl ester	58	.55	
Benzoyl-D-alanine methyl ester	24	.0073	c
Hexahydrobenzoyl-D-alanine methyl ester	Not a substrate		
Methyl furourate	37	1.17	d
Methyl tetrahydrofurourate	86	.143	d
Furoyl-L-alanine methyl ester	49	.51	d
Tetrahydrofuroyl-L-alanine methyl ester	132	.40	d
Furoyl-D-alanine methyl ester	19	.039	d
Tetrahydrofuroyl-D-alanine methyl ester	61	.017	d
Benzoyl-L-valine methyl ester	4.6	.045	e
Hexahydrobenzoyl-L-valine methyl ester	10	Insoluble above first order range	

TABLE XII (Cont'd)

Substrate	K_o, mM	k_o^b, sec.$^{-1}$	Ref.
Furoyl-L-phenylalanine methyl ester	.36	74	d
Tetrahydrofuroyl-L-phenylalanine methyl ester	1.2	95	d

a) In aqueous solution at 25°, pH 7.9 ± .1 and 0.1 M in sodium chloride.

b) Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

c) Unpublished experiments of W. A. Mukatis in these laboratories.

d) Part I, this thesis.

e) Waite, H. R. and Niemann, C., Biochem. 1, 250 (1962).

in k_o are irregular in direction but uniformly small. These results are readily explained by assuming a large diminution of the R_1 - ρ_1 interaction, upon reduction of the aromatic ring, with little change in the positioning of the substrate in the productive orientation.

These latter data may offer an explanation for the behavior of the aromatic-hexahydroaromatic pairs of inhibitors in Table XI. There is a pronounced similarity between Tables XI and XII with respect to the effect on K_o of reducing the aromatic ring. This similarity suggests that these inhibitors associate with the enzyme predominantly through a ρ_1 interaction rather than through a ρ_2 interaction as has previously been assumed (3). While this evidence is certainly far from conclusive, it is the only evidence presently available other than intuition, which bears on the orientation of the monofunctional inhibitor-enzyme interaction.

The data in Table XII also have an interesting bearing on the previously examined substrates having the ring in the amino acid side chain. One explanation offered, and subsequently rejected (4), for the observation that the values of K_o and k_o for acetyl-L-hexahydrophenylalanine methyl ester are lower than those for acetyl-L-phenylalanine methyl ester is that non-productive combination is being encountered with the latter substrate but not with the former. The most likely non-productive mode is the COR_3- ρ_3, R_2- ρ_1 orientation

However, the data in Table XII offer convincing evidence that hexahydroaromatic - ρ_1 interactions are certainly not favored over the corresponding aromatic interactions. Therefore, these data offer further cause for rejection of the above explanation.

2. Analogs of Known Substrates

Now that one can predict which R_i - ρ_i interactions will determine K_o and k_o for a given substrate with some degree of confidence (6a,b,c), it is of interest to examine the specificity, not of the enzyme-substrate interaction as a whole, but of the individual R_i - ρ_i interactions. For example, what structural specificity is exhibited by the ρ_2 locus? We know that acylated phenylalanine and tyrosine esters are among the best known substrates of alpha-chymotrypsin and that they are of the $S^{3E}_{R_2R_3}$* limit type. It is reasonable to assume that structural modifications of acetyl-L-phenylalanine methyl ester will result in a substrate of the same limit type unless the benzyl side chain is reduced to a very small side chain of the order of methyl or ethyl groups. By working with a limit case of this nature one can study the structural specificity of the individual ρ-sites. This, indeed, has been done to a limited degree.

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*Reference 6a. $S^{3E}_{R_2R_3}$ symbolizes a trifunctional ester type substrate with R_2 and COR_3 contributing to binding and $S^{3E}_{R_1R_3}$ symbolizes a similar type but with R_1 and COR_3 in binding roles.

One might wonder, for example, about the shape of the ρ_2-locus. Is it an area specifically suited for a benzyl or p-hydroxybenzyl group, or is it perhaps a longer groove extending out from the asymmetric carbon atom? Previous studies have been made which bear on the latter possibility but so far they are somewhat confusing. It has been shown that N-acetyl-L-tyrosine methyl ester has the same K_o but approximately twice as large a k_o as acetyl-L-phenylalanine methyl ester which differs only by lack of a para-hydroxy group (7). Etherification of the phenolic hydroxyl group results in a dramatic decrease in values of k_o and relatively small changes in K_o (7). It would appear then that introduction of something into the ρ_2 site just beyond the ring of phenylalanine markedly decreases the hydrolytic ability of the enzyme. However, N-acetyl-L-tryptophane methyl ester, which surely contains bulk in just that position described above, has been shown to have a k_o value in the range of those of phenylalanine and tyrosine methyl esters. Note that the above effects are due to substitution relatively far from the asymmetric carbon atom.

N-Acetyl-L-phenylglycine methyl ester, on the other hand, was studied to learn more about ρ_2 specificity near the asymmetric carbon. This substrate is a homolog of N-acetyl-L-phenylalanine methyl ester, the two differing by one methylene group. The kinetic constants for the former are K_o = 2.4 mM and k_o = 0.88 sec^{-1} while those for the

latter are 1.3 mM and 53 sec.$^{-1}$ (4). The small difference in K_o between the two shows that there is little difference in enzyme-substrate binding in the two cases. At the same time there is nearly a hundred-fold diminution of k_o when the phenyl ring is moved adjacent to the asymmetric carbon. This may be due to either, or both, of two effects. First, poor positioning of the carboalkoxy group with respect to the catalytic group (5) at the active site might be the cause of the low value for k_o. If this is the case, the poor positioning is achieved such that the overall enzyme-substrate interaction does not change substantially. While this is certainly possible, this selective positioning of the COR_3 group (where the COR_3 - ρ_3 interaction is not greatly altered but the hydrolytic action is greatly influenced) seems less likely than the following effect. Second, a steric hindrance to hydrolysis of the carbonyl function by branching of the side chain beta to the potentially reactive carbonyl group has been noted several times (6a,8,9).* This compound offers the first example of the beta-carbon being part of a phenyl ring and this difference appears to have an interesting consequence. In the previous example of beta-branching, methyl groups were the branching groups, and in

*Comparison of Corey models of acetyl-L-phenylalanine methyl ester with the corresponding valine, phenylglycine and cyclohexylglycine derivatives shows very limited access to the carbonyl carbon of the latter compounds relative to that of the phenylalanine substrate which has no beta-branching.

every case the diminution in k_o was accompanied by a large increase in K_o. If we look at acetyl-L-valine methyl ester, for example, where there is an isopropyl side chain, and two methyl groups beta to the carbonyl carbon, the value of K_o is 112 mM compared to K_o = 2.4 mM for the phenyl side chain. What is seen, then, is that aliphatic beta-branching gives steric hindrance both to binding and to hydrolysis, while aryl beta-branching hinders hydrolysis but not binding. We immediately wondered what the effect of reducing the ring to give acetyl-L-hexahydrophenylglycine methyl ester would be. Would K_o then approximate that of the valine compound or would it be more similar to that for the aromatic substrates? This question was answered when the values of K_o = 1.3 mM and k_o = 0.26 $sec.^{-1}$ were found. Again, there appears to be a steric hindrance to hydrolysis (k_o for acetyl-L-phenylalanine methyl ester is 53 $sec.^{-1}$) but none to binding. The remarkable difference in binding of the isopropyl and cyclohexyl side chains must be due to a positive binding interaction between the enzyme and the part of the cyclohexyl ring farthest down the chain from the carboalkoxy group.

One final point with regard to the substrate activity of acetyl-L-phenylglycine methyl ester. At the outset of this work there seemed to be little hope that the above compound would be a substrate because of the observation of Kaufmann and Neurath (10) that no substrate activity was

observed for ethyl-dl-alpha-benzamidophenyl acetate. Now that a moderate substrate activity has been observed for the acetyl compound we must explain these contradictory results for the benzoylated compound. Kaufmann and Neurath reported their results as 2-3% hydrolysis after 30 minutes in a 30% methanol solution. Applewhite, Martin and Niemann (11) have studied the effect of mixed solvent systems on the alpha-chymotrypsin-catalyzed hydrolysis of methyl hippurate. In their study they found that only a 10% methanol solution showed an eight-fold diminution in k_o and a three-fold increase in K_o relative to those constants in water. If one makes a linear extrapolation to the 30% methanol solution used by Kaufmann and Neurath then a twenty five-fold diminution in k_o and a nine-fold increase in K_o is expected. Besides this factor which would greatly slow down the reaction, a dl-mixture was used which would slow the reaction even more. If, then, the constants of Kaufmann and Neurath's benzoyl compound are similar to those of the moderate substrate, acetyl-L-phenylglycine methyl ester, as would be expected, it is no great surprise that the hydrolysis of ethyl-dl-alpha-benzamidophenyl acetate in 30% methanol was immeasurably slow.

We turn now to a few compounds closely related to, but distinctly different from, the usual acylated amino acid ester substrates of alpha-chymotrypsin.

Wolf (12) demonstrated that the rates of enzyme

TABLE XIII

Kinetic Constants for Two Pairs of Carbon-Nitrogen Analogs[a]

Substrate	K_o[b], mM	k_o, sec.$^{-1}$
Methyl-N-acetylglycinate[c]	14	.007
Methyl levulinate[c]	31	.015
Methyl-N-benzoylglycinate[d]	7.5	.20
Methyl beta-benzoylpropionate[e]	.29	.19

a) In aqueous solution at 25°, pH 7.9 and 0.5 M in sodium chloride unless otherwise noted.

b) Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

c) Wolf, J.P., III, and Niemann, C., Biochem. 2, 493 (1963).

d) Gordon, T. P. Ph.D. Thesis, California Institute of Technology, 1959.

e) Reaction solution 0.02 M in sodium chloride.

hydrolysis of methyl-N-acetylglycinate and methyl levulinate are very similar. This suggests that the requirements for substrate activity of bifunctional substrates are met equally well by a methylene group in place of the usual amino group. Since Wolf also determined the kinetic constants for methyl-N-benzoylglycinate, it was decided to examine its carbon analog, methyl-beta-benzoyl-propionate. The results of this, with the reference compounds, are listed in Table XIII. Again, k_o is the same for both the amido- and keto- compound. However, in the latter pair of substrates, K_o for the carbon analog is twenty-fold less than that for the amide. The insensitivity of k_o to replacing the amido NH group by a methylene group in these two pairs of substrates is, at first, somewhat surprising in view of the importance of the amido group in trifunctional substrates.

Neurath and Schwert (13) have proposed the necessity of participation by the alpha-amido group in hydrogen bonding of substrates to the enzyme. Kuk-Meiri and Lichtenstein (14) supported this view in explaining why they could see no alpha-chymotrypsin-catalyzed hydrolysis of alpha-N-methyl-L-phenylalanine ethyl ester. Recently, Niemann, et al. (7), have shown a decrease in reactivity of the order of 10^4 when the amido group of alpha-N-acetyl-L-tyrosine methyl ester is methylated. They point out that this evidence, as well as the preceding observations, can

be explained equally well on steric grounds as by the above hydrogen bonding theory. Whatever the reason, the necessity of the integrity of the alpha-amido group in trifunctional substrates has been fairly well established.

In Part I of this thesis we showed that aromatic, bifunctional substrates are hydrolyzed predominantly through $R_3 - \rho_3$, $R_1 - \rho_2$ interactions, while trifunctional L-substrates have previously been shown to be hydrolyzed through a $R_3 - \rho_3$, $R_1 - \rho_1$ enzyme-substrate complex (6a,b,c). The above results offer another illustration of the qualitative differences to be expected between bi- and trifunctional substrates because of their different productive orientations. Since these bifunctional substrates are hydrolyzed through a $R_1 - \rho_2$ rather than a $R_1 - \rho_1$ interaction, one would not expect the same structural specificity for the R_1 group found in trifunctional L-substrates. The significant difference in K_o values for the two benzoyl compounds, while there is no observable difference for the acetyl substrates, is probably due to an increased sensitivity to structure arising from the greater enzyme-substrate binding with the benzoyl derivatives.

During the course of the above work, it was decided to look for alpha-chymotrypsin-catalyzed hydrolysis of methyl benzoylacetate. This is not very closely related to the usual bifunctional substrates (derivatives of glycine) but it is the next lower homolog of methyl beta-benzoyl-

propionate. This compound turned out to be a slow substrate with constants of K_o = 1 mM and k_o = .047 sec.$^{-1}$. Since this substrate is only distantly related to the usual substrates derived from the amino acids, we wondered whether it was hydrolyzed at the same active center. To answer this question, the inhibition of methyl benzoylacetate by benzamide was studied. This inhibition was found to be fully competitive and a K_I of 5.5 ± 1 mM was found. This is just the effect of benzamide upon the enzyme-catalyzed hydrolysis of N-acetyl-L-valine methyl ester and N-acetyl-L-leucine methyl ester (15). Therefore, methyl benzoylacetate is hydrolyzed at the same active site as are bi- and trifunctional substrates. This compound serves to illustrate the remarkably broad range (the limits of which are not yet known) of substrates of alpha-chymotrypsin.

Another observation of Wolf and Niemann is that methyl-N-phenylglycinate is hydrolyzed by alpha-chymotrypsin at an easily measurable rate. This was surprising at the time because of the previously discussed observation of Kuk-Meiri and Lichtenstein (14) that N-alkyl phenylalanine esters show no substrate activity. The explanation of these differences between the bifunctional and trifunctional substrates was discussed above and is based upon the different binding modes leading to products in the two cases. We felt it would be worthwhile to look at the N-phenylalanine methyl esters for more proof of this explanation.

As it turned out, the rate of enzyme catalyzed hydrolysis of the dl-mixture was measurable, giving the constants K_o = 1.8 mM and k_o = .0035 sec.$^{-1}$. The two isomers were hydrolyzed at sufficiently similar rates that enzymic resolution was not feasible. We felt that the observed low order of reactivity was sufficient support for the above explanation, hence the resolution of the two isomers was not attempted.

The last compound studied here is related to the above discussion. We have assumed that N-phenylglycine methyl ester is hydrolyzed through a R_3 - ρ_3, R_1 - ρ_2 interaction. This, then, makes methyl-N-phenylglycinate an analog of L-phenylalanine methyl ester where the alpha-acylamido group is removed and we have an amino group replacing the methylene group of the phenylalanine side chain. Note that the benzyl side chain in phenylalanine derivatives is the R_2 group while we will call the phenyl-amino group in phenylglycine the R_1 group, yet both interact primarily with the ρ_2 locus of the enzyme. It has been shown that a substitution of a para-hydroxyl on the phenyl-alanine ring, giving N-acyl-L-tyrosine methyl ester, raises k_o substantially. A comparison of these two substrates is shown in Table XIV. If the methyl-N-phenylglycinate is an analog of phenylalanine methyl ester as we have suggested, then, one would expect that methyl-N-(para-hydroxyphenyl)-glycinate would have a substantially higher k_o than its

TABLE XIV

A Comparison of Benzyl and para-Hydroxybenzyl Side Chains with Phenylamino and para-Hydroxyphenylamino Side Chains[a]

Substrate	K_o, mM	k_o, sec.$^{-1}$	Ref.
N-Acetyl-L-phenylalanine methyl ester	1.3	53	(4)
N-Acetyl-L-tyrosine methyl ester	1	100	(7)
N-phenylglycine methyl ester[c]	.3	.0076	(12)
N-(para-hydroxyphenyl)glycine methyl ester[d]	2.5	.096	

a) In aqueous solution at 25°, pH 7.9 and 0.1 M in sodium chloride unless otherwise noted.

b) Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

c) Reaction system 0.5 M in sodium chloride.

c) Reaction system 0.02 M sodium chloride.

non-hydroxylated counterpart. That this is the case is shown in Table XIV.

It is noteworthy that there is a significant increase in K_o upon hydroxylation of the latter compound. This is easily understood on the basis of increased $R_1 - \rho_2$ and $R_1 - \rho_1$ binding in the <u>para</u>-hydroxylated compound. In the trifunctional pair there is probably no appreciable $R_2 - \rho_1$ binding in either compound.

D. EXPERIMENTAL*

(i) alpha-Chymotrypsin

The enzyme preparations used were the same as those in Part I of this thesis.

(ii) N-Acetyl-dl-phenylglycine methyl ester

alpha-Aminophenylacetic acid (dl) was esterified by the procedure of Brenner and Huber (16). The product, dl-phenylglycine methyl ester hydrochloride, was recrystallized from methanol-ether to give white crystals, m.p. 202-203° with decomposition. This compound was acetylated by the method of Applewhite, Waite and Niemann (17). Two recrystallizations from diisopropyl ether gave white microcrystals, m.p. 82-83°.

(iii) N-Acetyl-L-phenylglycine methyl ester

5 g. of the dl compound (ii) was taken up in 200 ml. water and the pH adjusted to 8.0. To this solution was added 100 mg alpha-chymotrypsin and .15 M NaOH as necessary to keep the pH constant at 7.9. After the reaction had gone to 48% completion (ca. 2 hrs.), the pH was adjusted to 10 and the reaction solution exhaustively extracted with

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*All melting points corrected by a calibration curve of Dr. A. Platt. All optical rotations were measured at room temperature, 25 ± 2° C., and have an uncertainty of ± 2-3%. Analyses are by Spang Microanalytical Laboratory, Ann Arbor, Michigan.

chloroform. The aqueous phase was then acidified to pH 1-2 and extracted with ethyl acetate. The ethyl acetate solution was dried over sodium sulfate and evaporated under vacuum. The tan residue, m.p. 198-199°, was taken up in methanol and again esterified. Four recrystallizations from isopropyl alcohol-diisopropyl ether gave colorless prisms, m.p. 115.5-116.5. $(\alpha)_D$ 174° (c. 5% in methanol).

N-acetyl-D-phenylglycine methyl ester was prepared from D-alpha-aminophenylacetic acid. m.p. 115.5-116.5°. $(\alpha)_D$ -173° (c. 5% in methanol).

Analysis:	calculated	C: 63.7	H: 6.3	N: 6.8
$C_{11}H_{13}O_3N$(207)	found	C: 63.7	H: 6.3	N: 6.9

(iv) N-Acetyl-L-hexahydrophenylglycine methyl ester.

The aromatic compound (iii) was reduced catalytically using platinum dioxide at 50 psi. H_2. Two recrystallizations of the product from diisopropyl ether gave colorless crystals, m.p. 117.5-118.5°. $(\alpha)_D$ 53.7° (c. 3% in chloroform).

Analysis:	calculated	C: 61.9	H: 8.9	N: 6.6
$C_{11}H_{19}O_3N$(213)	found	C: 61.8	H: 8.8	N: 6.7

(v) Hexahydro methyl hippurate

Methyl hippurate was reduced catalytically, as above. Three recrystallizations from ethyl acetate-hexane gave long needles, m.p. 100-101°. Lit. (18) m.p. 100-101°.

Analysis: calculated C: 60.3 H: 8.6 N: 7.0

$C_{10}H_{17}O_3N$(199) found C: 60.1 H: 8.7 N: 7.1

(vi) Hexahydrobenzoyl-L-alanine methyl ester

This was prepared by the catalytic reduction of benzoyl-L-alanine methyl ester. Two recrystallizations from diisopropyl ether gave colorless prisms, m.p. 91-92°. $(\alpha)_D$ -43.8° (c. 4% in methanol).

Analysis: calculated C: 61.9 H: 8.9 N: 6.6

$C_{11}H_{19}O_3N$(213) found C: 62.1 H: 8.9 N: 6.5

(vii) Hexahydrobenzoyl-D-alanine methyl ester

This was prepared in the same manner as the above compound, only the D-isomer was used as starting material. Two recrystallizations from diisopropyl ether gave colorless prisms, m.p. 91-92° $(\alpha)_D$ 44.0° (c. 4% in methanol).

(viii) Hexahydrobenzoyl-L-valine methyl ester

This was prepared by catalytic reduction of benzoyl-L-valine methyl ester. Two recrystallizations from diisopropyl ether gave colorless needles, m.p. 112.5-113.5°. $(\alpha)_D$ 16.5° (c. 4% in chloroform).

Analysis: calculated C: 64.7 H: 9.6 N: 5.8

$C_{13}H_{23}O_3N$(241) found C: 64.1 H: 9.8 N: 6.0

(ix) beta-Benzoyl methyl propionate

beta-Benzoylpropionic acid was prepared according to Martin and Fieser (19). This was esterified by the Brenner and Huber (16) method. The colorless liquid resulting was distilled twice at 70-75° and 10-15 μ Hg. The liquid solidifies in the refrigerator but melts well below room temperature. Lit. m.p. 18° (20).

Analysis:	calculated	C: 68.7	H: 6.3
$C_{11}H_{12}O_3$	found	C: 68.8	H: 6.4

(x) Benzoylacetic acid methyl ester

This was prepared from the ethyl ester by an ester interchange reaction. Two distillations at 115-117° and 1 mm. Hg gave a colorless liquid, $(n)_D^{25}$ 1.5370. Lit. b.p. 151-152° at 15 mm. Hg (21), and $(n)_D^{25}$ 1.5365 (22).

Analysis:	calculated	C: 67.5	H: 5.7
$C_{10}H_{10}O_3$(178)	found	C: 67.5	H: 5.7

(xi) N-(p-hydroxyphenyl)-glycine methyl ester

The procedure of Brenner and Huber (16) was employed to esterify N-(p-hydroxyphenyl)-glycine. Two recrystallizations of the product from methanol-diisopropyl ether gave colorless plates, m.p. 97-98°.

Analysis:	calculated	C: 59.7	H: 6.1	N: 7.7
$C_9H_{11}NO_3$(181)	found	C: 59.6	H: 6.2	N: 8.2

(xii) N-phenyl-dl-alanine methyl ester

To 42 g. aniline (0.45 mole) was added slowly 49.0 g. alpha-bromopropionic acid (0.32 mole). The resulting solid was taken up in 100 ml. absolute methanol and esterified according to the method of Brenner and Huber (16). After removal of solvent, the product was distilled, b.p. 115-120° and 1 mm. Hg. This liquid was redistilled twice giving 21 g. (37 percent theoretical yield) of pale yellow liquid $(n)_D^{25}$ 1.5333. The liquid crystallized upon refrigeration giving prisms which melt at 39-41°. Base hydrolysis gave a white compound melting at 159-161° unpurified. (N-phenyl-alanine melts at 163°.)

Analysis:	calculated	C: 67.0	H: 7.3	N: 7.8
$C_{10}H_{13}O_2N$	found	C: 67.0	H: 7.3	N: 8.1

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Part III

A FURTHER STUDY OF MONOFUNCTIONAL AROMATIC INHIBITORS OF ALPHA-CHYMOTRYPSIN

A. INTRODUCTION

While a great deal of effort has gone into the study of alpha-chymotrypsin catalysis as a function of various parameters, pH, ionic strength, nature of added salt, substrate, and temperature, the effect of added inhibitors had not gone far past the evaluation of acyl-D-amino esters until recently (1). Added inhibitors present a parameter, variable over a great range, whose full potential for yielding useful information is not yet known.

This study was initiated with a two-fold purpose: one, to continue the survey of Wallace, Kurtz and Niemann (1) of the simple aromatic inhibitors of alpha-chymotrypsin, and two, to study the combined effect of two inhibitors on alpha-chymotrypsin-catalyzed hydrolysis.

B. RESULTS

The results are summarized in Tables XV and XVI. The kinetic procedure was described in Part I of this thesis.

Some of the inhibition constants, K_I, were evaluated from the expression

$$K_I = v_o K_o (I)/(k_o(E)(S) - v_o(K_o + (S)))$$

where v_o is the initial velocity in the presence of inhibitor and K_o and k_o values are for the uninhibited substrate. This procedure allows evaluation of K_I from any number of kinetic runs at constant or varying substrate concentration. In other cases, K_I was evaluated from the expression

$$K_o' = K_o\left(1 + \frac{(I)}{K_I}\right)$$

where K_o' is the apparent Michaelis constant in the inhibited reaction system. This procedure requires several kinetic runs at constant inhibitor concentration and varying substrate concentrations. The values of K_I were taken as the enzyme-inhibitor dissociation constants for fully competitive inhibition unless otherwise noted.

Since calculation of inhibition constants involves the extraction of three kinetic constants from the same primary data usually used to obtain two kinetic constants, a lower degree of precision is usually encountered. This is reflected in the, sometimes substantial, errors attributed to values of K_I.

TABLE XV

Some Inhibitors of α-Chymotrypsin[a]

Inhibitor	(I), mM	(S), mM	(E)[b], M	No. Runs	K_I, mM	Value of Wallace, Kurtz and Niemann (1)
Benzene[c]	8.0	16-112	40	4	25 ± 3	
Toluene	3.5	16-112	40	4	13 ± 1	
Nitrobenzene	1	16-109	41	8	4.4± .4	
Benzonitrile	1.4	16-109	41	6	0.8± .2	
Acetophenone	2.2	16-109	42	7	2.1± .2	
α-Tolunitrile	2.7	16-109	42	8	1 ± .5	
Chlorobenzene	1.8-3.1	16, 32	40	5	3.3± .3	
Fluorobenzene	5.1-9.0	16, 32	40	5	5.0± .7	
Halotoluenes[d]	Too insoluble					
Aniline	4.0	17-120	40	7	7.3± .5	6.6
Aniline[e]	8.0	4.3- 30	18	7	7.5± 1.7	
Anisole	2.0	17-120	40	7	7.4± 1	8.4
Benzenesulfonamide	2.1	17-123	40	5	5.5± 1	4.3
Quinoline[e]	.45	30- 21	40	7	0.76±.07	0.6

TABLE XV (Cont'd)

Inhibitor	(I),mM	(S),mM	(E)[b], M	No. Runs	K_I,mM	Value of Wallace, Kurtz and Niemann (1)
Tetracyanoquinone dimethane[d]		Too insoluble				
Cyclohexane	7	16,32	40	No observable inhibition		
Benzylammonium ion	1.2	17-123	40	4	26 ± 5	
Cyclohexylammonium ion	6.8-7.9	16	40	2	128 ± 30	
Acetonitrile	140	16, 32	40	(No observable inhibition)		
Acetyl-D-phenylglycine methyl ester	3.1	16-109	42	5	19 ± 8	
Acetyl-D-hexahydrophenyl-glycine methyl ester[f]	8.0	1.3-9.1	3.6	8	28 ± 9	

a) In aqueous solution at 25°, pH 7.9 and 0.1 M in sodium chloride.

b) Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme.

c) Inhibitors are evaluated against acetyl-L-valine methyl ester unless otherwise noted.

d) Solubility is less than 1 mM.

e) Evaluated against methyl hippurate.

f) Evaluated against acetyl-L-hexahydrophenylglycine methyl ester.

TABLE XVI

Inhibition of Acetyl-L-Valine Methyl Ester by Mixtures of Inhibitors[a]

Aniline, mM	Anisole, mM	K_o' experimental[b]	K_o' calculated[c]	$(K_o'exp.-K_o'calc.)/K_o'exp. \times 100$
4.04	2.03	.19	.20	-5
12.1	6.09	.29	.38	-31
4.04	12.2	.51	.51	0
16.1	6.09	.44	.41	7
12.1	8.13	.46	.43	6
20.2	4.06	.41	.38	7
8.07	10.2	.49	.47	4
24.2	2.03	.36	.35	3
16.1	4.06	.36	.35	3
8.07	8.13	.44	.41	7
12.1	4.06	.32	.32	0
8.07	6.09	.36	.35	3
Benzenesulfonamide, mM	Benzylammonium ion, mM			
2.11	6.75	.29	.27	7
4.22	5.63	.23	.29	-27

TABLE XVI (Cont'd)

Benzenesulfonamide, mM	Benzylammonium ion, mM	K_o' experimental[b]	K_o' calculated[c]	$(K_o'exp.-K_o'calc.)/K_o'exp. \times 100$
2.11	5.63	.24	.25	-4
2.11	4.50	.23	.23	0
4.22	4.50	.28	.28	0
6.33	4.50	.36	.33	8

a) In aqueous solution at 25°, pH 7.9 and 0.1 M in sodium chloride.

b) Calculated from the expression $v = k_o(E)(S)/((S) + K_o')$.

c) If one assumes simple additivity of inhibition by the two inhibitors, the following expression for K_o' is found, $K_o'_{calc.} = K_o\left(1 + \frac{(I_1)}{K_{I_1}} + \frac{(I_2)}{K_{I_2}}\right)$, where K_o is the measured Michaelis constant in the absence of inhibitor, K_I and K_{I_2} are the inhibition constants found for each inhibitor individually.

C. DISCUSSION

This work was initiated to look for inhibition by benzene and toluene. Drs. R.A. Wallace and A.N. Kurtz, in these laboratories, had just completed a broad survey of inhibition by aromatic compounds. The inhibition constants of all of the uncharged, monosubstituted benzenes they studied were very similar, having K_I values of approximately 8 mM. These results suggested to us that the observed inhibition was a function of the aromatic ring and not due to the small polar substituent. In order to show this, the inhibition constants of benzene and toluene were evaluated. The K_I values found were 25 and 13 mM respectively. That these values are somewhat higher than those for the other uncharged, monosubstituted aromatics shows that while the benzene ring alone is sufficient for inhibition, a substantial increase in inhibitory activity is imparted by the addition of a relatively polar group on the ring.

A review of the study by Wallace, Kurtz and Niemann (1) showed that, with the exception of the aromatic carboxamides, all of the monosubstituted benzenoid compounds they examined contained electron releasing substituents. We, therefore, examined the inhibitory activity of another half dozen aromatics containing electron attracting substituents. Reference to these compounds in Table XV, compounds three

through eight, shows that K_I values for these compounds tend to be slightly lower than for those containing electron donating substituents. K_I values for the monosubstituted aromatic inhibitors studied by Wallace, Kurtz and Niemann average approximately 8 mM while the average for these benzenoid inhibitors with electron attracting substituents is nearer 3 mM.

In order to see of the above differences were real, and not due to differences in experimental technique, the next four compounds, aniline through quinoline, were examined. The value of K_I for each of these compounds was found to be comparable to that value found by Wallace, et al. (1). We conclude that benzene rings containing electron attracting substituents are, in general, slightly better inhibitors of alpha-chymotrypsin than are benzene rings containing electron releasing substituents.

A disubstituted benzene, para-nitrophenol, containing both a strong electron releasing group and a strong electron donating group was examined, and a K_I value of 7 mM found. It is significant that this value is comparable to that of either phenol (6.4) or nitrobenzene (4.4) rather than significantly lower than K_I for either monosubstituted aromatic. This latter possibility would have indicated additivity of the effects of the two substituents.

In order to test whether π-acid-π-base interactions are important in enzyme-inhibitor complexes, an attempt was

made to evaluate the inhibition constant of tetracyanoquinone dimethane, a very strong π-acid. This compound proved too insoluble to allow evaluation of its inhibitory activity.

The difference in K_I values for phenol and cyclohexanol, 6.4 mM and 75 mM respectively, led Wallace, Kurtz and Niemann (1) to their first postulate: "For interactions at a single and common locus aromatic compounds are more effective inhibitors than their hydroaromatic analogues." The present study includes two more pairs of aromatic-hexahydroaromatic analogs which support the above postulate. While benzene has an inhibition constant of 25 mM, no inhibition was observed for cyclohexane at a concentration of 7 mM, signifying that K_I must be greater than 70 mM. Likewise cyclohexylammonium ion gave a K_I of 128 mM while its aromatic counterpart, benzylammonium ion, gave a K_I of 26 mM.

Acetonitrile was examined because water-acetonitrile mixed solvent systems have been used for substrates which are too insoluble to be studied in water. That this mixed solvent system does have an effect upon the observed kinetic constants of methyl hippurate has been shown by Applewhite, Martin and Niemann (2). However, the effect does not show up as a competitive inhibition with the trifunctional substrate used here.

The last two compounds in Table XV were prepared

during the synthesis of their L-isomers for Part II of this thesis. Their inhibition constants were evaluated in case they might be of future interest.

It is well known that a broad spectrum of aromatic compounds combine with the active site of alpha-chymotrypsin and inhibit the reactions catalyzed by this enzyme. The possibility that not all of these inhibitors combine with the same portion of the active site has never been explored. In fact, one does now know what portion of the active site (ρ_1, ρ_2, or ρ_3) is involved in any enzyme-inhibitor interaction. This latter problem is much more difficult to deal with than the former problem and probably will have to await the answer to whether all aromatic inhibitors interact with the same ρ-site.

An approach to the question of whether all inhibitors interact with the same locus of the enzyme lies in the study of reaction systems containing two inhibitors. If the inhibitors each show fully competitive inhibition against the substrate individually and if they each interact with the same portion of the enzyme surface, then one would expect the two inhibitors to compete with each other for the enzyme. The following formulae illustrate this situation:

$$E + S \rightleftharpoons ES \rightarrow E + P$$

$$E + I_1 \rightleftharpoons EI_1$$

$$E + I_2 \rightleftharpoons EI_2$$

This situation can easily be shown to give rise to the rate expression:

$$v = \frac{-d(S)}{dt} = \frac{d(P)}{dt} = \frac{k_o(E_1)(S)}{(S) + K'_o} \quad , \quad K'_o = K_o\left(1 + \frac{(I_1)}{K_1} + \frac{(I_2)}{K_2}\right)$$

where $K_o = \frac{(E)(S)}{(S)}$, $K_1 = \frac{(E)(I_1)}{(EI_1)}$, and $K_2 = \frac{(E)(I_2}{(EI_2)}$.

This is simply additivity of the effects of two competitive inhibitors.

If, on the other hand, these two fully competitive inhibitors interact with different portions of the active site then they will not compete with one another when mixed and one would expect the added equilibrium:

$$EI_1 + I_2 \rightleftarrows EI_1I_2 \rightleftarrows EI_2 + I_1$$

in which a ternary complex is formed between the enzyme and both inhibitors. In the latter case the rate expression is of a different nature:

$$\frac{dP}{dt} = \frac{k_o(E)(S)}{S + K'_o} \quad , \quad K'_o = K_o\left(1 + \frac{K_1}{(I_1)}\right)\left(\frac{K_2 + (I_2)}{1 - K_2}\right)$$

These two rate equations do not illustrate all possibilities of course, as the two inhibitors could interact with each other, or with the substrate, but judicious selection of the compounds will avoid these latter effects and allow one to distinguish whether the two inhibitors are fully competitive with each other.

It is essential for this type of study that one know the precise nature of inhibition by each inhibitor individually. It need not be fully competitive inhibition; however, this type of inhibition allows the least cumbersome system for initial experiments.

The results of the preliminary experiments conducted in this study are summarized in Table XVI.

Prior to the study of mixtures of aniline and anisole, these two inhibitors were evaluated individually under the same conditions and found to show fully competitive inhibition, with the K_I values recorded in Table XV. For the first study of this type, two similar inhibitors were selected with the intention of finding simple additivity of inhibition by the two inhibitors. This hope was realized and the correlation of K_o' calculated with K_o' experimental, with the one exception, is considered very good.

Now that this approach has been shown to work, there will be a great deal of effort spent in this direction. As was stated at the beginning, the goal is to find inhibitors which combine with the enzyme at different loci. This search will most likely be successful if one studies mixtures of inhibitors of widely differing structure. For example, studies should include mixtures of charged and uncharged inhibitors, aromatic and aliphatic inhibitors, benzenoid and heteroaromatic compounds such as indole.

An experiment of this type was attempted in the study of mixtures of benzenesulfonamide and benzylammonium ion.

These data, with the omission of the second set, show a good correlation of experimental and calculated values for K_o' assuming simple additivity of the effects of two competitive inhibitors.

Future studies in this direction offer a great potential for the understanding of inhibition of alpha-chymotrypsin. The inhibitors, in turn, are the most convenient probes at our disposal for delineation of the active site of α-chymotrypsin.

D. EXPERIMENTAL

(i) alpha-Chymotrypsin

The enzyme preparations used were the same as those in Part I of this thesis.

(ii) Inhibitors

All inhibitors used in this study were either recrystallized three times or, if liquid, were distilled twice under nitrogen.

(iii) N-Acetyl-L-valine methyl ester

This substrate was prepared in the same way as that of Applewhite, Waite and Niemann (3).

(iv) Methyl picolinate

This compound was prepared by the procedure of Furst and Jelesaroff (4).

(v) Acetyl-D-phenylglycine methyl ester

D-(-)-alpha-Phenylglycine (Aldrich) was esterified by the method of Brenner and Huber (5). This methyl ester hydrochloride has a melting point 201-202°. The acetylation was carried out just as on (iii). Three recrystallizations from isopropyl alcohol-diisopropyl ether gave colorless prisms, m.p. 116-117°. $(\alpha)_D^{25}$ -173 ± 1° (c. 5% in methanol).

Analysis:	calculated	C: 63.7	H: 6.3	N: 6.8
$C_{11}H_{13}NO_3$(207)	found	C: 63.6	H: 6.3	N: 7.1

(vi) Acetyl-D-hexahydrophenylglycine methyl ester

A mixture of 2.5 g. acetyl-D-phenylglycine methyl ester, 1 g. platinum oxide and 60 ml. of glacial acetic acid was put under 55 psi. H_2 for 12 hours. The solution was filtered and the acetic acid removed under reduced pressure. The resulting solid was recrystallized twice from diisopropyl ether to give 1.6 g. (64% theoretical yield) of colorless needles, m.p. 117-118°. $(\alpha)_D^{25}$ -53.0 ± .5° (c. 3% in chloroform).

Analysis:	calculated	C: 61.9	H: 8.9	N: 6.6
$C_{11}H_{19}NO_3$(213)	found	C: 61.9	H: 8.9	N: 6.7

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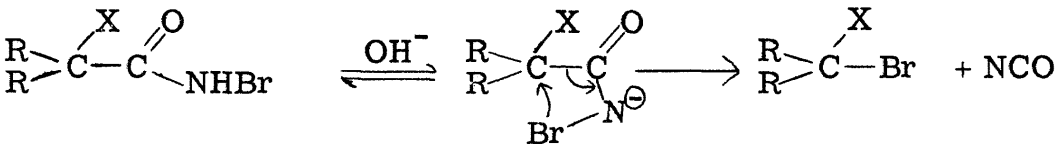
PROPOSITION I

A study of the Lossen rearrangement is proposed where an electronegative group is substituted alpha to the hydroxamate group. The hydroxylamine used will be substituted with ^{18}O. Examination of the resulting ketone, with respect to isotopic oxygen, will allow evaluation of the reaction mechanism.

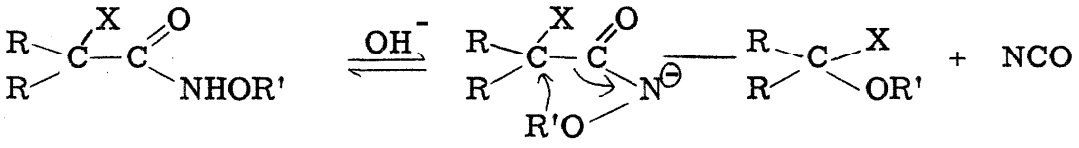
In a recent series of papers, Stevens, et. al. (1 a, b, c), have examined the conversion of α-haloamides to gem-dihalides under conditions similar to those employed in the Hoffman rearrangement. The reaction is found to be exclusively intramolecular and to proceed with a high degree of stereospecificity.

An examination of the reaction scheme suggests that a similar



rearrangement might be observed with α-halohydroxamic acid derivatives.

An attempt to observe the following rearrangement (similar to the reaction studied by Stevens, et. al.) is proposed.



Such a rearrangement would be easily overlooked as it would generally give the same final products as would the Lossen rearrangement itself.

An easy method for assaying the amount of the sought-for reaction

is the use of hydroxylamine-^{18}O in forming the hydroxamate. Thus, intramolecular attack on the alpha carbon atom by a nitrene-like nitrogen atom will allow isolation of unlabelled ketone, while attack by the oxygen atom will give rise to ^{18}O-ketone.

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PROPOSITION II

It is proposed that N-benzyl-D-alanine methyl ester will be a substrate for alpha-chymotrypsin while the L-isomer will not. This will constitute the first specific example where an inversion of the usual antipodal specificity was predicted prior to its discovery.

It has been firmly established that the stereospecificity of alpha-chymotrypsin is relative rather than absolute (1). Indeed, three examples of inversion of the usual antipodal specificity, i.e., three cases where the enzyme catalyzed hydrolysis of the D-antipode is faster than that of its L-isomer, have been found (1a,2,3). These inversions were explained by a general theory developed by Hein and Niemann (1b,c) and by the much more limited, but very similar, theory of Cohen, et al. (2).

In a review in 1950, Neurath and Schwert (4) proposed the necessity of participation by the alpha-amido group in hydrogen bonding of substrates to the enzyme. Kuk-Meiri and Lichtenstein (5) supported this view when they found that while N-acyl-L-phenylalanine methyl esters are among the fastest known substrates of alpha-chymotrypsin, there are no observable enzyme-catalyzed hydrolysis of N-alkyl-L-phenylalanine ethyl esters. Recently, Peterson, Hubele and Niemann (6), have shown a decrease in reactivity of the order of 10^4 when the amido group of alpha-N-acetyl-L-tyrosine methyl ester is methylated. They point out that this evidence, as well as the preceding observations, can be explained equally

well on steric grounds as by the hydrogen bonding theory.

Whatever the cause, the necessity of the integrity of the alpha-amido group in trifunctional L-substrates has been established. Observe that the observations were made on $S^{3E}_{R_2R_3}$.* type substrates so that one might further conclude that the requirement for the integrity of the alpha-amido group lies in the specificity of the ρ_1-locus.

The observation by Wolf and Niemann (7), then, that N-phenyl methyl glycinate is a substrate of alpha-chymotrypsin, came as somewhat of a surprise. The explanation for this is offered in Part I of this thesis where it is shown that aromatic bifunctional substrates are hydrolyzed predominantly through an R_3 - ρ_3, R_1 - ρ_2 orientation. This orientation does not place the alpha-N-alkyl group into the ρ_1 -locus which has been shown to cause loss of substrate activity.

In Part II of this thesis it was shown that N-phenyl-dl-alanine methyl ester functioned as a substrate of alpha-chymotrypsin, having the kinetic constants K_o = 1.8 mM and k_o = .0035 sec.$^{-1}$. It was noted that enzyme resolution was not possible because no sharp break in reaction rate was observed at 50% hydrolysis. This is to be expected, even if the L-compound is not a substrate, because the reaction

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*Reference 1b. $S^{3E}_{R_2R_3}$ symbolizes a trifunctional ester type substrate with R_2 and COR_3 contributing to binding and $S^{3E}_{R_1R_3}$ symbolizes a similar type but with R_1 and COR_3 in binding roles.

velocity is so close to that of the non-enzyme-catalyzed hydrolysis.

It has been shown already that positioning of an N-alkyl group into the ρ_1-locus, as happens with L-substrates, causes loss of substrate activity. It was shown by the glycine derivative that substrate activity is not necessarily lost if there is a productive orientation open to the substrate which avoids the $R_1 - \rho_1$ interaction. That such is the case of D-alanine methyl ester was shown in great detail in Part I of this thesis. Therefore, the substrate activity of the above compound must be due to the D-isomer.

For experimental convenience, demonstration of the above explanation should be carried out with the stereoisomers of N-benzyl-alanine methyl ester.

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PROPOSITION III

It is proposed that a dilute solution of optically active n-propylbenzene-α -d-ring-^{14}C in benzene be equilibrated in the presence of gallium bromide and hydrogen bromide (in the manner of the Streitwieser and Reif (3) experiment on ethylbenzene). Examination for radioactivity, optical rotation and deuterium composition of aliquots of this solution during equilibration will contribute a great deal toward the understanding of this reaction.

Disproportionation and isomerization of alkylbenzenes upon treatment with strong Lewis acids have long been known and studied (1). Many conflicting reports about these reactions have been resolved since the development of better analytical techniques, namely infrared spectroscopy and gas chromatography. Nevertheless, the only case where the mechanism of the reaction has been elucidated is that of ethylbenzene which is simplified by the observation of Roberts, Ropp and Neville (2) that no ethyl isomerization occurs.

Our understanding of the disproportionation of ethylbenzene is due to the elegant study of Streitwieser and Reif (3). These workers suggested the following mechanism to explain their results:

$$C_6H_5CH_2CH_3 \overset{*R^{\oplus}}{\rightleftharpoons} C_6H_5\,CHCH_3 \overset{C_6H_5}{\rightleftharpoons} C_6H_5 - \underset{C_6H_5}{CHCH_3} \longrightarrow$$

*The original cations presumably arise from traces of styrene present initially and from progressive oxidation during the reaction.

$$C_6H_5 + C_6H_5\overset{\oplus}{C}HCH_3 \xrightarrow[\text{rate determining}]{C_6H_5CH_2CH_3} C_6H_5CH_2CH_3 + C_6H_5\overset{\oplus}{C}HCH_3$$

A great deal of study has gone into the Lewis acid catalyzed equilibration of the propylbenzenes, yet this reaction remains far less clear than that of ethylbenzene. Actually, the isomerization and disproportionation of the butyl and pentylbenzenes have been investigated also. It is apparent that an understanding of these latter reactions is based upon an understanding of the mechanism of the propylbenzene rearrangement.

A brief summary of the data regarding the treatment of n-propylbenzene with Lewis acids is given below.

1. n-propylbenzene, under the influence of strong Lewis acids and hydrogen halide, disproportionates to give benzene and dipropylbenzene, the latter being converted slowly to tripropylbenzene (4).
2. The propylbenzene fraction from an equilibration contains 2-5% isopropylbenzene (5).
3. The n-propylbenzene fraction from an equilibration of n-propylbenzene-beta-^{14}C shows complete equilibration of the carbon atoms alpha and beta to the ring. No label is affixed to the gamma position (6,7).
4. Examination of the isopropylbenzene fraction from the tagged equilibration shows 50% of the label alpha to the ring and 50% beta to the ring (5).

While several mechanisms have been proposed to explain these data, they can be grouped into two classes: 1) those requiring protonation of the ring and subsequent disproportionation and/or isomerization of the alkyl group (4,6,8) and, 2) those involving initial abstraction of a hydride ion from the alkyl side chain (3,5,9).

It is proposed that a dilute solution of optically active n-propylbenzene-α-d-ring-^{14}C in benzene be equilibrated in the presence of gallium bromide and hydrogen bromide (in the manner of the Streitwieser and Reif (3) experiment on ethylbenzene). Examination for radioactivity, optical rotation and deuterium composition of aliquots of this solution during equilibration will distinguish between the above two classes of mechanisms and will contribute a great deal toward an understanding of this reaction.

The mechanisms which start with abstraction of a hydride (deuteride) ion from the side chain of n-propylbenzene predict formation of both d_o and d_2 n-propylbenzene, while those mechanisms, the initial step of which is ring protonation, will not allow such deuterium scrambling.

The triple labelling technique allows a comparison of the rates of loss of radioactivity, loss of optical activity and deuterium scrambling. This will yield a great deal of knowledge about the mechanism within the general class as determined above.

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PROPOSITION IV

A study of the reactivity of boron monochloride in a microwave discharge tube is proposed.

Boron monochloride has been observed spectrally as an intermediate in the microwave discharge synthesis of diboron tetrachloride from boron trichloride (1). An emission spectrogram of the light in the 2200 Å to 7000 Å range produced during the discharge showed only bands identified as arising from BCl.

Volpin, et al. (2), have predicted that elements other than carbon whose electron valent shells are similar to carbene may be expected to exhibit reactivity similar to that of carbenes. BCl is a compound of this type, and as such is expected to form new covalent bonds readily, as do carbenes.

It is proposed to trap this intermediate by forming it in the presence of carbon-hydrogen bonds and carbon-carbon multiple bonds where insertion and addition reactions may be expected to occur.

The greatest difficulty facing this study lies in the unknown stability, or instability, of both the trapping agents and the products formed. It may prove necessary to sweep the BCl formed into the trapping agent.

In an attempt to circumvent this problem a saturated hydrocarbon, 2,2,4-trimethylpentane, is suggested as the first trapping agent. Insertion of carbene into this compound has been observed several times (3,4). The appropriate blank reaction should be carried out.

An even more interesting reaction will be that of BCl with cyclohexene, in the manner of Kopecky, Hammond and Leermakers (4). Provided the substrate is sufficiently stable under the reaction conditions, this reaction will allow a comparison of the reactivity of BCl in insertion and addition reactions.

These studies may lead to the synthesis of a new heteroaromatic compound, borirene.

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PROPOSITION V

It is proposed that methoxyamine be used, in place of hydroxylamine, as a water analog in the chymotrypsin-catalyzed hydrolysis of acetyltyrosine ethyl ester. This will allow evaluation of the importance of the hydroxyl group, with particular reference to the possible formation of o-acetyltyrosylhydroxylamine as an intermediate.

The question of an acyl-enzyme intermediate in the α-chymotrypsin-catalyzed hydrolysis of substrates derived from α-amino acids has aroused a recent interest in the effect of hydroxylamine on these reactions (1-6). A great deal of the present confusion results from the simultaneous observations of Epand and Wilson (4), Caplow and Jencks (5) and Bender, et al. (6), that the fraction of acetyltyrosine hydroxamic acid produced during the chymotrypsin-catalyzed hydrolysis of acetyltyrosine ethyl ester in the presence of hydroxylamine is dependent upon the concentration of enzyme used.

These observations have been explained by Epand and Wilson (4) and by Kezdy, Clement and Bender (6) on the basis of equilibrium between acyl-enzyme + hydroxylamine and enzyme + O-acylhydroxylamine where the O-acylhydroxylamine can give hydroxamic acid by a non-enzymic route (7). A similar explanation could be given with the equilibrium involving a ternary complex rather than an acyl-enzyme. Thus, neither the acyl-enzyme mechanism nor that of Bernhard, Coles and Nowell (1) is favored by this type of explanation.

Caplow and Jencks (5) have suggested that aggregation of the enzyme at higher concentrations is a possible explanation of the results but they hasten to add that they have no experimental evidence bearing on the possibility of such aggregation.

It has been shown that O-acylation of hydroxylamine is negligible in the non-enzyme-catalyzed hydroxylaminalysis of simple esters (7). Caplow and Jencks (3) have shown that furoyl-chymotrypsin in the presence of 0.025 M hydroxylamine gives largely O-furoylhydroxylamine.

The substitution of methoxyamine for hydroxylamine, as a water analog, is proposed. This will allow evaluation of the importance of the hydroxyl group in the enzyme-dependent product ratio observed in the competitive hydrolysis and hydroxylaminolysis of acetyltyrosine ethyl ester.

If the same enzyme-dependence is observed, obviously O-acylation of the hydroxylamine cannot be the explanation. The absence of an enzyme-dependence when methoxyamine is used will support, but not prove, the intermediacy of O-acylhydroxylamine.

If the O-acylation of hydroxylamine is supported by the above experiment, an attempt should be made to trap the O-acylhydroxylamine by working at low concentrations of hydroxylamine so that the intermediate will not be converted non-enzymatically to hydroxamic acid so fast as to preclude isolation of it.

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