THE ALPHA-CHYMOTRYPSIN-CATALYZED HYDROLYSES OF DERIVATIVES OF N-ACETYL-L-PHENYLALANINE METHYL ESTER AND OF METHYL HIPPURATE

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ABSTRACT

N-Acetyl-L-phenylalanine methyl ester and 23 of its derivatives (all substituted at the phenyl ring of the parent compound) have been evaluated as substrates of α-chymotrypsin. The results are interpreted in terms of a model of the active site of the enzyme. An elaboration of the model for the locus of the active site where the side chains of the amino acid derivatives are bound is offered.

The rates of the α-chymotrypsin-catalyzed hydrolyses of N-acetyl-N-methyl-L-tyrosine methyl ester and of methyl para-hydroxyhydrocinnamate were evaluated. Comparisons among the kinetic constants for these reactions and for the α-chymotrypsin-catalyzed hydrolyses of N-acetyl-L-tyrosine methyl ester are made. The effect of the structural modifications of the acetylamino group of N-acetyl-L-tyrosine methyl ester on the kinetic constants of its α-chymotrypsin-catalyzed reaction are again interpreted in terms of a model of the active site of α-chymotrypsin.

Finally, four derivatives of methyl hippurate were evaluated as substrates of α-chymotrypsin. Two other derivatives of methyl hippurate were reversible competitive inhibitors of α-chymotrypsin. All six were substituted on the benzene ring of the benzoylamino group. The results are compatible with the proposal that the benzamido groups of methyl hippurate derivatives interact with the same locus of the enzymatic active site as do the side chains of derivatives of N-acetyl-L-phenylalanine methyl ester.

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INTRODUCTION

Enzymes are proteins which can catalyze certain chemical reactions (1, 2, 3). One characteristic property of enzymes as a class of catalysts is their structural and stereochemical specificity (1, 4). An understanding of this phenomenon is, therefore, a vital part of the larger problem of describing the chemical nature and mechanism of action of enzymes.

α-Chymotrypsin and its Substrates

The enzyme, α-chymotrypsin is an endopeptidase; it catalyzes the hydrolysis of protein peptide bonds inside the peptide chain (1, 5, 6). It also catalyzes the hydrolysis of a large number of synthetic compounds, whose most common structural feature is the carboxyl group and its functional derivatives (1). In catalyzing the hydrolysis of both these classes of compounds, α-chymotrypsin exhibits a relative and broad structural specificity.

Undoubtedly the most extensively studied group of synthetic substrates of α-chymotrypsin consists of derivatives of acylated α-amino acids (1, 5, 6). These substrates may serve as models for the natural protein substrates, since the chemical structures of the former incorporate four functional groups in common with the latter. They are: 1) the hydrolyzable carboxyl derivative, 2) the acylamino function, 3) the side chain, and 4) the hydrogen atom adjacent to the carboxyl derivative. Except for glycine derivatives, these four groups are disposed about an asymmetric center in both classes of compounds.

One advantage of the simple model compounds over proteins as substrates is that they give only two products upon hydrolysis. The rates of the enzyme-catalyzed hydrolyses of model substrates can therefore be measured with relative ease, and quantitative data is made readily accessible. Only qualitative information is available from proteins because the number of products from protein hydrolysis makes quantitative kinetic measurements extremely difficult (1, 7).

The Kinetics of α-Chymotrypsin-catalyzed Hydrolyses

The rates of many α-chymotrypsin-catalyzed hydrolyses obey the equation:

$$-\frac{d[S]}{dt} = \frac{d[P]}{dt} = \frac{ko[E]o[S]}{Ko + [S]} \qquad 1$$

The concentrations of substrate, product, and total enzyme are given by [S], [P], and [E]o, respectively. The terms ko and Ko are kinetic parameters characteristic for each substrate at a given set of experimental conditions (i.e., pH, temperature, solution composition, etc.).

The simplest kinetic scheme which fits equation 1 is the following (8):

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

A steady-state treatment of this kinetic scheme leads to a rate equation identical in form to equation 1, with $k_o = k_2$ and $K_o = (k_{-1} + k_2)/k_1$ (8). The formation of the enzyme-substrate complex (ES) in the course of many enzyme-catalyzed reactions, including those catalyzed by α-chymotrypsin, has been well established experimentally (9-16). The constant Ko is most simply interpreted as an index of a substrate's ability to combine with the enzyme to form an ES complex. The constant ko is interpreted as the rate with which the enzyme-substrate complex proceeds to products and uncomplexed enzyme.

The Structural Specificity of α-Chymotrypsin-catalyzed Hydrolyses

The results from studies of acylated α-amino acid derivatives as substrates of α-chymotrypsin are in accord with studies of its proteolytic activity with respect to side chain specificity (1, 5, 6, 7). The highest frequency of scission of peptide bonds in the presence of α-chymotrypsin occurs at phenylalanyl, tyrosyl and tryptophanyl bonds (1, 6, 7). In a series of strictly comparable acylated α-amino acid derivatives (e. g., acetylated L-α-amino acid amides or the corresponding methyl esters),the highest ko values and the lowest Ko values are observed for L-phenylalanine, L-tyrosine and L-tryptophan derivatives (5, 6). Thus, all other factors being equal, the side chain specificity of α-chymotrypsin for both its natural and model substrates is for benzyl, para-hydroxybenzyl, and β-indolylmethyl side chains.

Comparison of the α-chymotrypsin-catalyzed hydrolyses of two acetylated α-amino acid esters with those of their benzoylated analogues and examination of a large body of acylated glycine esters

as substrates of the enzyme reveals that α-chymotrypsin can exhibit a specificity for the acyl function of its model substrates (2, 6, 17, 18). Substitution of the acyl group of N-acetyl-L-alanine methyl ester by a benzoyl moiety lowers the Ko by a factor of approximately 60 (6, 17). The analogous replacement in N-acetyl-L-valine methyl ester decreases its Ko by a factor of 20 (6, 17). At the same time, the ko's of both benzoylated compounds decrease by a factor of four compared to their acetyl analogues (6, 17). Substitution of benzoyl, phenacetyl, and para-aminobenzoyl for the acetyl group of acetylglycine methyl ester uniformly lowers the Ko of its α-chymotrypsin-catalyzed hydrolysis by a factor of two (18). The ko's of the first and third compounds increase while that of the second compound is unchanged, compared to the acetyl analogue (18).

Replacement of the acetyl group of acetyl-D-alanine methyl ester by a benzoyl group converts a compound with no measurable susceptibility to α-chymotrypsin-catalyzed hydrolysis to one with low, but measurable activity (6, 17). The Ko is lower by a third than that of its L enantiomer, while the ko is lower by a factor of 20 than the ko of the L compound (6, 17). Thus the stereochemical, as well as structural, specificity of model substrates in α-chymotrypsin-catalyzed reactions has been shown to be relative (6, 17).

The Hein-Niemann Theory

A theory of the structural specificity of α-chymotrypsin proposed by Hein and Niemann provides an explanation for the data accumulated from studies of the hydrolysis of many model substrates, including those just discussed, in the presence of this enzyme (17).

This theory successfully correlates all the known kinetic data from hydrolysis of model substrates collected under comparable conditions (6, 17, 18a, b). It may be used to predict certain results and one prediction has been confirmed (19).

Both a definition and a model of the active site of α-chymotrypsin had been proposed when the study of acyl α-amino acid derivatives as model substrates of the enzyme was in its early stages (20, 21). That definition and that model were assumed in the development of the Hein-Niemann theory (17). The postulated model of α-chymotrypsin's active site (defined as that portion of the enzyme where hydrolysis of proteins, peptides, and the model substrates occurs) is an asymmetric arrangement of limited flexibility of three loci designated ρ_1, ρ_2, and ρ_3 (20, 21). The locus ρ_3 is assigned the two-fold function of catalyzing the hydrolytic scission of the hydrolyzable group (COR_3) and of binding it during the catalytic process. Under optimum conditions the side chain (R_2) is assumed to bound at ρ_2 and the acylamino group (R_1) at ρ_1. A fourth locus, ρ_H, is postulated (primarily for the sake of convenience) to indicate the space occupied by the α-hydrogen when R_1-ρ_1, R_2-ρ_2 and COR_3-ρ_3 interactions occur.

According to the Hein-Niemann theory, any R_i-ρ_j interaction (where the i and j are not necessarily identical subscripts) is feasible, with the restriction that only one substrate molecule can combine with the active site at a time. Thus several enzyme-substrate binding modes are possible for each substrate. Those binding modes not featuring COR_3-ρ_3 interactions will necessarily be incapable of proceeding to products and are fully competitive with those which can. Hein and Niemann showed that a kinetic treatment of this concept of fully competitive binding modes produces

a rate equation of the same form as the Michaelis-Menten-Henri rate law for enzyme-catalyzed reactions (equation 1) (17).

By assigning relative stabilities to each of the several possible binding modes and relative reactivities to each of the potentially productive complexes, Hein and Niemann were able to use the derived rate equation to correlate the available data with the hypothetical model. The conclusions these authors reached from the correlations can be summarized in the statements which follow.

1) For the interactions of model substrates with α-chymotrypsin, carboalkoxy groups are better bound at ρ_3 and are more susceptible to hydrolysis when bound at ρ_3 than are amide groups.

2) The ρ_2 locus preferentially interacts with bulky substituents (e. g., phenyl rings) on any of the three groups (R_1, R_2 and R_3) surrounding the carbon adjacent to the carboxyl group derivative. A binding mode featuring such an interaction has a lower Ko than one which does not.

3) The interaction of a group as large as, or larger than, a methyl group with the ρ_H locus does not contribute positively to the binding energy of an enzyme-substrate interaction and inhibits the hydrolysis of the enzyme-substrate complex.

4) An R_1 group must be bound at ρ_1 to achieve optimum values of ko. The function of this interaction is to induce spatial relationships between the COR_3 group and the catalytic groups in ρ_3 such that optimum catalytic activity is achieved.

5) Branching in a substrate side chain at positions two carbons removed from the carbonyl of the carboxyl derivative of the substrate sterically hinders both binding and hydrolysis.

These statements are consistent with the behaviour of the substrates mentioned earlier. The benzoyl derivatives of L-alanine and L-valine methyl esters have lower Ko values than the corresponding acetyl derivatives because of the presence of the bulky phenyl ring (see statement 1)). The binding mode incorporating the benzoylamino-ρ_2 interaction is catalytically inactive, however, because it places a group larger than a methyl in ρ_H (see statement 3)). The benzoyl-D-alanine methyl ester has substrate activity because it can bind to the enzyme in a mode which incorporates the catalytically necessary COR_3-ρ_3 interaction, which places a bulky group in ρ_2 and which does not place a group as large as a methyl in ρ_H. The rate of the α-chymotrypsin-catalyzed hydrolysis of benzoyl-D-alanine methyl ester is still slower than its enantiomer at least partially because an R_1-ρ_1 interaction has been lost.

Present Investigations

The investigations reported in this thesis concern three major classes of compounds as substrates of α-chymotrypsin.

The first class of compounds whose kinetic parameters as substrates of α-chymotrypsin were determined is made up of 23 derivatives of N-acetyl-L-phenylalanine methyl ester, in which substituents have been placed on the phenyl ring (see Figure 1a). Comparisons of their kinetic parameters to those of the parent compound and among themselves make possible an evaluation of the

effect of extending the side chain dimensions on binding of the substrate to the enzyme and on susceptibility to hydrolysis of the resulting enzyme-substrate complexes. The study may be interpreted as an exploration of the ρ_2 locus of the model active site used by Hein and Niemann in their theory of the structural specificity of α-chymotrypsin-catalyzed reactions (17).

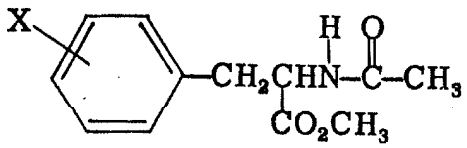


Figure Ia. General derivative of N-acetyl-L-phenylalanine methyl ester.

The second class of compounds consists of N-acetyl-L-tyrosine methyl ester (Figure 1a, X = p-OH) and two analogs modified at the acylamino function. In one of the analogs, the hydrogen on the nitrogen of the acetylamino function has been replaced by a methyl group. In the other, the entire acylamino group has been replaced by a hydrogen. Comparisons of the kinetic parameters of their α-chymotrypsin-catalyzed hydrolyses along with those of certain other substrates permit an assessment of the role assigned by Hein and Niemann to the acylamino group of α-chymotrypsin's model substrates.

The last class of compounds whose behaviour in the presence of α-chymotrypsin was investigated contains six derivatives of methyl hippurate (see Figure 1b) in which substituents have been placed on the phenyl ring of the parent compound's benzoyl group. An extensive study of the effect of varying the acyl group of several acylated glycine, D-alanine, and L-alanine methyl esters on the kinetics of their α-chymotrypsin-catalyzed hydrolyses was made by J. R. Rapp (19, 22). The results of his investigation demonstrated the soundness of the interpretation that R_1-ρ_2 interactions can occur during the α-chymotrypsin-catalyzed hydrolysis of certain substrates. However, except for the benzoyl derivatives of the amino acid esters he investigated, none of the acyl functions could be directly compared to side chains of other acylamino acid esters. Such comparisons would aid assessments of the relative frequency of R_1-ρ_1 interactions compared to R_1-ρ_2 interactions of acylated glycine, D-alanine, and L-alanine methyl esters in their interactions with α-chymotrypsin (17, 18a, b, 22). The reports given in this thesis of the abilities of six methyl hippurate derivatives substituted on the phenyl ring to function as substrates or inhibitors of α-chymotrypsin and of the substrate activity of the analogous derivatives of N-acetyl-L-phenylalanine methyl ester make a limited number of such comparisons possible.

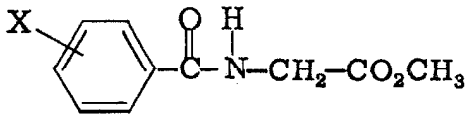


Figure 1b. General derivative of methyl hippurate.

RESULTS

The rates of the α-chymotrypsin-catalyzed hydrolyses of the acylated α-amino acid esters evaluated in this study may be described by the familiar Michaelis-Menten-Henri rate law (equation 1 of the introduction). All kinetic determinations reported in this thesis were conducted in aqueous solutions at 25.0°, pH 7.9, and 0.10M in sodium chloride.

N-Acetyl-L-phenylalanine Methyl Ester and its Derivatives

The kinetic parameters for N-acetyl-L-phenylalanine methyl ester and 23 of its derivatives as substrates of α-chymotrypsin-catalyzed hydrolysis are presented in Table I.

The values of ko and Ko for the hydrolysis of N-acetyl-L-phenylalanine methyl ester in the presence of α-chymotrypsin have been reported (23, 24). The values reported by Jones and Niemann are ko = (52 ± 16)sec^{-1} and Ko = (1.2 ± 1.0) x 10^{-3}M (23). Their evaluation was performed in an aqueous medium at 25.0°, pH 7.9, and 0.10M in sodium chloride. Bender and Glasson evaluated the kinetic constants of this reaction in aqueous solutions at 25.2°, pH 7.8, 0.042M in sodium chloride, and 7.4 x 10^{-4}M in tris-(hydroxymethyl)-aminomethane (24). They report values of Ko = (1.80 ± 0.45) x 10^{-3}M and ko = (63 ± 16)sec^{-1} (when α-chymotrypsin is assumed to have a molecular weight of 25,000 and a protein-nitrogen constant of 16.5%) (23, 24). Decreasing sodium chloride concentration usually lowers the ko values and increases the Ko values in the α-chymotrypsin-catalyzed hydrolyses of methyl ester substrates (1, 20, 27, 33).

TABLE I: α-Chymotrypsin-catalyzed Hydrolyses of Several Derivatives of N-Acetyl-L-phenylalanine Methyl Ester[a]

X[b]	ko[c]	Ko[d]	ko/Ko[e]
no substituent	67 ± 2	0.62 ± 0.06	108
ortho-fluoro	76 ± 7	0.78 ± 0.12	97
ortho-chloro	15 ± 1	0.54 ± 0.08	28
ortho-hydroxy	11 ± 3	2.4 ± 0.8	4.6
ortho-methoxy	3.74 ± .03	0.093 ± 0.021	40
ortho-ethoxy	3.8 ± 0.3	1.3 ± 0.2	2.9
meta-fluoro	130 ± 25	0.53 ± 0.14	245
meta-chloro	120 ± 10	0.092 ± 0.018	1,300
meta-methyl	77 ± 2	0.32 ± 0.04	240
meta-nitro	380 ± 50	0.37 ± 0.10	1,030
meta-hydroxy	245 ± 54	10.2 ± 2.5	24
meta-methoxy	250 ± 60	4.7 ± 1.6	53
meta-ethoxy	19 ± 1	22 ± 2	0.86
meta-isopropoxy	208 ± 23	11 ± 2	19
3,5-dimethoxy[f]	150 ± 100	25 ± 17	6.1
para-fluoro	64 ± 3	0.48 ± 0.04	130
para-chloro	13 ± 1	0.17 ± 0.03	76
para-methyl	27 ± 1	1.0 ± 0.1	27
para-nitro	18 ± 2	0.42 ± 0.09	43
para-amino	118 ± 13	3.0 ± 0.5	39
para-hydroxy	117 ± 7	0.32 ± 0.04	370
para-methoxy	0.49 ± 0.03	2.6 ± 0.4	0.19
para-ethoxy	10 ± 1	12 ± 2	0.83
para-isopropoxy	0.012 ± 0.005	1.6 ± 0.4	0.0075

a) In aqueous solutions at 25.0°, pH 7.90 ± 0.10, 0.10M in sodium chloride.

b) Refers to the substituent and its position on the phenyl nucleus of N-acetyl-L-phenylalanine methyl ester relative to the β methylene bridge.

c) In units of sec^{-1}.

d) In units of $10^{-3}M$.

e) In units of $10^{-3}M^{-1}sec^{-1}$.

f) The precision is low because of insolubility of the substrate; only orders of magnitudes of the constants are significant.

If this observation is kept in mind, the reported values and values in Table II are in good agreement.

No report of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine methyl ester (the para-hydroxy derivative in Table I) has ever appeared in the literature. However, the ethyl ester has been described as a substrate for α-chymotrypsin and the pertinent parameters have been reported (25, 26). Provided the kinetic determinations are performed under strictly comparable conditions, the parameters for the α-chymotrypsin-catalyzed hydrolysis of the two esters should be nearly identical (6, 17). Cunningham and Brown report $k_o = (197 \pm 7)\ sec^{-1}$ and $K_o = (0.070 \pm 0.05) \times 10^{-3}M$ for the ethyl ester as a substrate of α-chymotrypsin in aqueous solutions at 25°, pH 8.0, $6.7 \times 10^{-3}M$ tris-(hydroxy-methyl)-aminomethane and, 0.10M in calcium chloride (25). It has been shown that replacement of sodium chloride by calcium chloride as an added electrolyte in α-chymotrypsin-catalyzed hydrolyses can increase the k_o value with only a small effect on K_o (27). The magnitude of the effect on k_o is somewhat dependent on the nature of the substrate structure (1).

TABLE II: Experimental Concentrations for α-Chymotrypsin-catalyzed Hydrolyses of Derivatives of N-Acetyl-L-phenylalanine Methyl Ester[a]

X[b]	$[S]_o$[c]			$[E]_o$[d]
no substituent	0.240	-	2.88	0.365
ortho-fluoro	0.107	-	0.959	0.211
ortho-chloro	0.121	-	1.09	0.796
ortho-hydroxy	0.110	-	0.877	0.522
ortho-methoxy	0.174	-	3.49	4.71
ortho-ethoxy	0.198	-	3.16	4.94
meta-fluoro	0.0460	-	0.368	0.109
meta-chloro	0.0280	-	0.224	0.109
meta-methyl	0.0928	-	2.60	0.147
meta-nitro	0.0845	-	0.760	0.109
meta-hydroxy	0.300	-	4.84	0.510
meta-methoxy	0.495	-	3.96	0.092
meta-ethoxy	1.92	-	34.5	4.86
meta-isopropoxy	1.11	-	8.90	0.113
3,5-dimethoxy	0.372	-	3.35	1.28
para-fluoro	0.0723	-	0.651	0.211
para-chloro	0.0462	-	0.416	5.31
para-methyl	0.306	-	2.76	0.365
para-nitro	0.0732	-	0.659	0.464
para-amino	0.216	-	3.89	0.175
para-hydroxy	0.0704	-	0.842	0.113
para-methoxy	0.781	-	6.25	14.8
para-ethoxy	1.06	-	8.44	3.15
para-isopropoxy	0.161	-	1.29	349

a) In aqueous solutions at 25.0°, pH 7.90 ± 0.10 and 0.10M sodium chloride.

b) Refers to the substituent and its position on the phenyl nucleus of N-acetyl-L-phenylalanine methyl ester, with respect to the β-methylene bridge.

c) In units of 10^{-3}M.

d) In units of 10^{-7}M, based on a molecular weight of 25,000 and a protein-nitrogen content of 16.5% for α-chymotrypsin.

The values of Cunningham and Brown are therefore in good agreement with those in Table I. Schwert and Kaufman report ko = 189 sec^{-1} and Ko = 32 x 10^{-3}M for the kinetic parameters for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester in a 30% (v/v) methanol-water system at 25°, an apparent pH of 8.0, and at an unspecified concentration of phosphate buffer (26). The effect of added methanol at concentrations of 10% (v/v) methanol in aqueous solutions at 25.0°, pH 7.9, and 0.02M in sodium chloride increased the Ko by a factor of 2.6 and decreased the ko by a factor of 6.6 relative to those constants obtained under the same conditions without the methanol for a methyl hippurate-α-chymotrypsin system (28). Comparisons of the values of Schwert and Kaufman to those of Cunningham and Brown or to those in Table I do not seem justified.

The O-alkylated derivatives of N-acetyl-L-tyrosine methyl ester have been reported as substrates of α-chymotrypsin in a communication which was a part of the studies presented in this thesis (29).

The experimental concentrations of enzyme and of substrate used in the determination of the kinetic parameters for the α-chymotrypsin-catalyzed hydrolysis of each of the compounds in Table I are listed in Table II.

N-Acetyl-L-tyrosine Methyl Ester and Two Analogs

Table III lists the kinetic parameters for the α-chymotrypsin-catalyzed hydrolyses of N-acetyl-L-tyrosine methyl ester and two of its structural analogs.

As discussed earlier, the kinetic parameters of the α-chymotrypsin-catalyzed hydrolysis of the ethyl ester of N-acetyl-L-tyrosine, but not the methyl ester, have been reported in the literature (25, 26).

The values for the kinetic constants for N-acetyl-N-methyl-L-tyrosine methyl ester as a substrate of α-chymotrypsin have been reported previously (29).

Methyl hydrocinnamate has been reported as a substrate of α-chymotrypsin-catalyzed hydrolysis (30, 31). Although constants were reported in both cases, 20% (v/v) methanol-water solutions were used in both kinetic determinations (30, 31). Consequently, comparisons between the reported kinetic constants for this substrate and the kinetic constants for its para-hydroxy derivative given in Table III are not valid.

Methyl Hippurate and its Derivatives

The kinetic parameters for the α-chymotrypsin-catalyzed hydrolyses of methyl hippurate and four of its derivatives are presented in Table IV. The inhibition constants of two derivatives of methyl hippurate evaluated as competitive inhibitors of N-acetyl-L-leucine methyl ester are also included. The para-ethoxy derivative was evaluated as both a competitive inhibitor of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-leucine methyl ester and as a substrate because its low substrate activity prevented accurate evaluation of its Ko value.

TABLE III: N-Acetyl-L-Tyrosine Methyl Ester and Two Analogs as Substrates in α-Chymotrypsin-catalyzed Hydrolysis[a]

Compound	$[S]_o$[b]	$[E]_o$[c]	k_o[d]	K_o[b]
N-acetyl-L-tyrosine methyl ester	0.0704 - 0.842	1.13	118 ± 8	0.32 ± 0.04
N-acetyl-N-methyl-L-tyrosine methyl ester	1.48 - 11.9	10,040	0.026 ± 0.004	8.4 ± 1.8
methyl para-hydroxy-hydrocinnamate	0.117 - 1.40	80.5	0.63 ± 0.07	1.0 ± 0.2

a) In aqueous solutions at 25.0°, pH 7.90 ± 0.10, 0.10M in sodium chloride.

b) In units of 10^{-3}M.

c) In units of 10^{-8}M, based on a molecular weight of 25,000 and a protein nitrogen content of 16.5%.

d) In units of sec^{-1}.

TABLE IV: Derivatives of Methyl Hippurate as Substrates or Inhibitors in α-Chymotrypsin-catalyzed Hydrolysis[a]

X[b]	ko[c]	Ko[d]	K_I[d]
no substituent[e]	0.22	5.3	-
para-amino[f]	0.0267 ± 0.0009	2.7 ± 0.9	-
meta-methoxy[g]	0.23 ± 0.04	3.1 ± 0.9	-
meta-ethoxy[h]	0.0045 ± 0.0007	3.8 ± 1.5	-
para-methoxy[i, j]	-	-	0.58 ± 0.06
para-ethoxy[j, k]	0.0023 ± 0.0015	6 ± 6	3.8 ± 0.7
para-isopropoxy[i, j]	-	-	16 ±10

a) In aqueous solutions at 25.0°, pH 7.90 ± 0.10, 0.10M in sodium chloride.

b) Refers to the substituent and its position relative to the benzamido carbonyl.

c) In units of sec^{-1}.

d) In units of 10^{-3}M.

e) Evaluated by J. R. Rapp (22).

f) [S]o = 0.944 to 18.9 mM, [E]o = 3.62×10^{-5}M, (see footnote 1).

g) [S]o = 0.615 to 4.92 mM, [E]o = 1.94×10^{-6}M, (see footnote 1).

h) [S]o = 1.29 +0 10.3 mM, [E]o = 1.41×10^{-4}M, (see footnote 1).

i) Has no detectable substrate activity with $[E]_o = 1.4 \times 10^{-4}$M.

j) Evaluated as a competitive inhibitor of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-leucine methyl ester.

k) $[S]_o = 1.14 - 4.54$ mM, $[E]_o = 1.36 \times 10^{-4}$M (see footnote l).

l) All enzyme molarities based on a molecular weight of 25,000 and a protein-nitrogen content of 16.5% for α-chymotrypsin.

Two of the substrates in Table IV had previously been evaluated as substrates of α-chymotrypsin (18, 22, 28). For methyl hippurate, Applewhite *et al.* report $k_o = 0.200$ sec^{-1} and $K_o = 7.6 \times 10^{-3}$M (28). Their kinetic determination was performed in the same reaction medium as that employed by Rapp, except the medium was 0.02M in sodium chloride (28) and not 0.10M in sodium chloride, as was Rapp's (22). Wolf and Niemann (18) report the kinetic constants for methyl para-aminohippurate as $k_o = 0.041$ sec^{-1} and $K_o = 8.3 \times 10^{-3}$M in aqueous solutions, 0.50M in sodium chloride. All the reaction media employed in this investigation were 0.10M in sodium chloride. It has been shown that with increasing concentration of sodium chloride in these reaction systems, the magnitude of k_o for the α-chymotrypsin-catalyzed hydrolyses of acylated amino acid esters steadily increases while K_o decreases to a point, then remains invariant (1, 27, 32, 33). The values of the kinetic constants for methyl hippurate follow both these trends (22, 28). In the case of methyl para-aminohippurate, the values of k_o follow

this trend but those of Ko do not. Because the values of the kinetic constants are within the same order of magnitude and because the range of substrate concentrations used in the present investigation is wide enough to detect a Ko of the magnitude of 8×10^{-3}M, it appears that the value of the Ko for methyl para-aminohippurate determined in this investigation is acceptable, even though it does not follow the trend established previously (1, 27, 32, 33). The other values in Table IV have not been reported previously.

Table V includes the values for the inhibited and uninhibited α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-leucine methyl ester from which the inhibition constants for the three para-alkoxy substituted methyl hippurates were calculated. The values of the kinetic constants for the α-chymotrypsin-catalyzed hydrolyses of N-acetyl-L-leucine methyl ester have been determined previously (34). The values determined by Hein *et al.* are ko = (4.6 ± 0.3) sec^{-1} and Ko = (2.9 ± 0.3) $\times 10^{-3}$M (34). These values agree well with the mean values of the uninhibited hydrolyses presented in Table V, i.e., ko = (5.3 ± 0.4) sec^{-1} and Ko = (3.2 ± 0.3) $\times 10^{-3}$M. In all except one case (the second value of the para-ethoxy case) the uncertainties in the ko values are large, so that the ko values are probably consistent with the uninhibited values. They are certainly of the same order of magnitude as the uninhibited ko's, so that the inhibition by the substituted methyl hippurates may be defined as fully competitive. The one case noted in the para-ethoxy experiments is apparently an anomaly.

If competitive inhibition is assumed, the inhibition constants may be calculated using the relation

$$K_I = [I]\, Ko/(Ko' - Ko) \qquad 2$$

TABLE V: Determination of Inhibition Constants of Derivatives of Methyl Hippurate, with N-Acetyl-L-Leucine Methyl Ester as Substrate[a]

X[b]	[S]o[c]	[E]o[d]	[I][c]	ko[e,f]	Ko[c,f]	K_I[g]
para-methoxy	0.225 - 7.28	0.766	-	5.1 ± 0.2	3.3 ± 0.2	-
para-methoxy	1.24 - 17.4	0.766	1.798	6.2 ± 2.3	13.4 ± 0.9	0.58 ± 0.06
para-methoxy[h]	1.24 - 17.4	0.766	5.394	8.7 ± 3.3	42 ±18	0.46 ± 0.22
para-ethoxy	0.343 - 4.39	0.940	-	5.1 ± 0.1	3.1 ± 0.1	-
para-ethoxy	1.50 - 24.1	0.940	3.294	6.0 ± 0.1	5.4 ± 0.3	4.6 ± 0.7
para-ethoxy[i]	1.50 - 24.1	0.940	4.392	7.0 ± 4.0	7.9 ± 1.1	3.0 ± 0.7
para-isopropoxy	0.520 - 5.54	0.922	-	5.7 ± 0.2	3.1 ± 0.2	-
para-isopropoxy[j]	0.862 - 6.89	0.922	3.474	5.9 ± 0.3	3.7 ± 0.4	16 ±10

a) In aqueous solutions at 25.0°, pH 7.90 ± 0.10 and 0.10M in sodium chloride.

b) Refers to the substituent and its position relative to the benzamido carbonyl.

c) In units of 10^{-3}M.

d) In units of 10^{-6}M, based on a molecular weight of 25,000 and a protein-nitrogen content of 16.5%.

e) In units of sec^{-1}.

f) Hein, Jones and Niemann report ko = (4.6 ± 0.3) sec^{-1} and Ko = (2.9 ± 0.3) $x10^{-3}$M (34).

g) The formulae used for calculation of the K_I values and their uncertainties are given in the text of the Results section.

h) The K_I with the lower uncertainty was chosen.

i) The arithmetic mean of the two calculated K_I's was chosen as the representative value.

j) Methyl p-isopropoxyhippurate has a low solubility in water.

where Ko is the binding constant of the substrate in the absence of inhibitor, Ko' is the apparent binding constant determined for the inhibitor in the presence of the inhibitor at a constant concentration [I], K_I is the binding constant of the inhibitor and [I] is the concentration of the inhibitor. The values of Ko and Ko' in the absence and presence of inhibitor at constant concentration were calculated by the method described previously (35). The K_I's were calculated from the formula given above and the uncertainties estimated by the relation

$$e = \sqrt{\left(\frac{\sigma}{Ko}\right)^2 + \frac{\sigma^2 + (\sigma')^2}{(Ko)^2 + (Ko')^2}} \qquad 3$$

where e is the uncertainty in $K_I\,[I]^{-1}$, σ is the uncertainty calculated for Ko, σ' is the uncertainty in Ko', and the definitions of Ko, Ko' and [I] are those given above. The formula for e is adapted from one given by Daniels *et al* (36).

The inhibition constant chosen for the para-methoxy case was the one with the lower uncertainty value. The inhibition constant for the para-ethoxy substituted methyl hippurate is the arithmetic mean of the two calculated values. The large uncertainty in the inhibition constant for methyl para-isopropoxy-hippurate was a result of the low solubility of this compound in water.

DISCUSSION

The Effective Concentration of α-Chymotrypsin in Dilute Solutions

Table II lists the concentrations of enzyme and of substrate used in the determinations of the kinetic parameters for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-phenylalanine methyl ester and 23 of its derivatives. The enzyme concentrations used in these determinations varied from 0.91×10^{-8}M to 5.3×10^{-7}M (assuming a molecular weight of 25,000 and a protein-nitrogen content of 16.5% for pure α-chymotrypsin) for all of these derivatives except the para-methoxy and the para-isopropoxy cases. In those cases, the enzyme concentrations were 1.5×10^{-6}M and 3.5×10^{-5}M, respectively.

It has been shown that at enzyme concentrations of the order of 10^{-7}M or lower, α-chymotrypsin is adsorbed on the glass surfaces of volumetric equipment and reaction cells to such an extent that the concentration of enzyme decreases significantly (37, 38). Bixler and Niemann concluded from their investigations of this phenomenon that when the concentration of the enzyme in the reaction cell is 5×10^{-6}M or higher, no significant decrease in enzyme concentration by adsorption on glass is observed (37). They added the enzyme to the reaction system by injecting a 1.0 ml. aliquot of an aqueous enzyme stock solution whose pH had been adjusted to 7.9 into a 9.0 ml. volume containing the aqueous solution of the other components of the reaction solution, also at pH 7.9 (37). Attempts to perform similar 1:10 dilutions on aqueous stock solutions 10^{-6}M or less in α-chymotrypsin resulted in large decreases in the apparent enzyme

concentrations, while a 1:100 dilution of an aqueous stock solution 5×10^{-5}M in α-chymotrypsin led to no apparent decrease in the enzyme concentration (37).

As Bixler and Niemann note, two sources of glass surface contribute to the adsorption of the enzyme from solution in the kinetic procedure used in these laboratories (37). First, the glass volumetric equipment in which aqueous stock solutions of α-chymotrypsin are prepared and stored, the vessels in which the pH of the stock solutions is adjusted, and the syringe used to transfer the enzyme to the reaction vessel all could deplete the concentration of the enzyme before it is introduced into the reaction vessel. The reaction cell itself is the other source of α-chymotrypsin-adsorbing surface. (Since the electrodes and stirrer have a surface area much smaller than that of the vessel walls and base, their contributions to the adsorbing surface can be neglected.)

In the kinetic determinations reported in this thesis, all enzyme stock solutions were never less concentrated than 10^{-5}M in α-chymotrypsin. When concentrations on the order of 10^{-8}M to 10^{-7}M α-chymotrypsin were needed in the reaction solution, thousand-fold dilutions of the aqueous stock solutions were made. Furthermore, at such high dilutions, adjustment of the stock solution pH prior to injection into the reaction vessel was unnecessary. The syringe used to transfer the enzyme stock solution to the reaction cell was rinsed several times with the stock solution prior to delivery. Thus, the possibility of significant losses of α-chymotrypsin from a stock solution prior to its introduction into the reaction solutions was eliminated.

Adsorption of α-chymotrypsin on the reaction vessels used in kinetic measurements cannot be completely eliminated. The alternative use of containers made of plastic materials may not eliminate the problem, since it is known that α-chymotrypsin tends to collect at hydrophobic surfaces, such as the interface between water and n-octadecane (40). Furthermore, adsorption of α-chymotrypsin from 10^{-8}M solutions on hydrophobic phases destroys its catalytic activity, while it retains some of its activity on glass surfaces (37-40).

The rate of diffusion of α-chymotrypsin from aqueous solutions to glass surfaces has been studied by Trurnit (38). Trurnit studied this phenomenon by measuring the rate at which the thickness of a layer of α-chymotrypsin on a glass surface increased with time. His experiments were carried out at 20^{o}, pH 6.9 to 8.7, and in buffer solutions up to 0.03M in sodium veronal (38). Trurnit found that the diffusion coefficient for α-chymotrypsin measured in aqueous solutions 0.20M in sodium chloride by Schwert and Kaufman was consistent with his results (41). Furthermore, Creeth has shown that the diffusion coefficient of serum albumin was constant in aqueous solutions whose ionic strength varied from 0.05M to 0.20M (42). Creeth also showed that the result was the same whether the ionic species were contributed by sodium chloride or sodium veronal (42). Trurnit found that there was no specific interaction of sodium veronal with α-chymotrypsin (38). These observations taken together indicate that the differences in the concentrations and types of added electrolyte species between Trurnit's systems and the ones employed in the kinetic determinations reported in this thesis

should have no effect on the rate with which α-chymotrypsin is adsorbed on the glass surface.

The temperature at which kinetic determinations were made in the studies reported herein was 25°, while Trurnit measured adsorption rates at 20° (38). The energy of activation for the adsorption of α-chymotrypsin is 5.1 ± 0.2 k. cal./mole (38). From this value, it may be computed that at 25° the rate of adsorption is no more than 1.2 times that at 20°.

The monolayer of α-chymotrypsin which forms on glass surfaces from its aqueous solutions at concentrations of 4×10^{-7}M or less has a density and thickness corresponding to 4.8×10^{-4} mg. α-chymotrypsin/cm.^{2} of glass surfaces (38). The 20 ml. beakers used as reaction vessels in the present investigations have a glass surface area in contact with the solution of 20-25 cm.^{2} when they contain a volume of 10 ml. solution (see Appendix 1). A maximum concentration change of 5×10^{-8}M α-chymotrypsin can be computed from these data.

The results of Trurnit's measurements of adsorption rates of α-chymotrypsin on glass surfaces may be applied directly to the systems in which the kinetic measurements reported in this thesis were made, since it has been shown in the preceeding discussion that the diffusion rates of α-chymotrypsin in the solutions of both investigations should be nearly identical. The concentrations of α-chymotrypsin used in Trurnit's investigation overlapped those in the kinetic measurements reported in this thesis, and both systems were at nearly the same pH (8.1 and 7.9, respectively).

Calculations of the rates of adsorption of α-chymotrypsin on the reaction cell walls under the experimental conditions

employed in the present study (see the first Appendix) show that the rate of diffusion of α-chymotrypsin out of solution should never lower the enzyme concentration to a value less than approximately 70% of its initial value over the eight minute interval needed to complete a kinetic run. Furthermore, the decrease in enzyme concentration over the first two minutes is never more than 10% of the initial enzyme concentration.

The computations of the kinetic parameters for the α-chymotrypsin-catalyzed hydrolyses of the acylated amino esters reported in this thesis were performed on the Burroughs 220 digital computer programmed by a method described previously (43). Since this program involves fitting the curve of the production of acid as a function of time to a fifth degree polynomial in time and finding the first derivative at zero time, the 10% decrease in enzyme concentration over the initial two minute interval should introduce no more than a 10% error in the computed initial velocities. It will be assumed that the concentrations of enzyme in the systems used to evaluate the kinetics of the α-chymotrypsin-catalyzed hydrolysis of the compounds listed in Tables I and II are accurate to within those limits. The close agreement of the values of ko and Ko determined for N-acetyl-L-phenylalanine methyl ester in the present study with those reported in the literature (see the Results section) supports this view.

The Effective Concentration of α-Chymotrypsin in Concentrated Solutions

The meta- and para-ethoxyhippuric acid methyl esters are such poor substrates of α-chymotrypsin that enzyme concentrations

of the order of 10^{-4}M were necessary for the determination of their ko and Ko values (see Table IV). There is a tendency for the enzyme to dimerize at concentrations above approximately 10^{-3}M in aqueous solutions at pH 7.9 and 0.30M in sodium chloride (44). This dimer is catalytically inactive with respect to esterase activity (44, 45). It will be assumed that the α-chymotrypsin solutions used in determining the kinetic parameters of these methyl hippurate derivatives contain only negligible concentrations of enzyme dimers, because the concentration of sodium chloride is lower and because the enzyme concentration is an order of magnitude lower.

The Configurations of the Derivatives of N-Acetyl-L-Phenylalanine Methyl Ester

All the derivatives of N-acetyl-L-phenylalanine methyl ester listed in Table I and Table II are assigned the L configuration. Of these compounds, the parent compound and its para-hydroxy derivative were prepared by the esterification and acetylation of the corresponding L-amino acids. The para-methoxy, para-ethoxy, and para-isopropoxy derivatives were prepared by esterification of the corresponding acids which had in turn been prepared from N-acetyl-L-tyrosine by methods which do not racemize or invert the configuration of this compound (29, 46). Nitration of phenylalanine, under the conditions employed in this investigation, also does not lead to racemization or inversion of configuration (47). Since the para-amino derivative was prepared by catalytic hydrogenation of N-acetyl-para-nitro-L-phenylalanine methyl ester, it too may be

assumed to be a pure L isomer. The parent compound and the last six of its derivatives in Table I possess unambiguously the L configuration.

Of the remaining compounds listed in Table I, all except the meta-hydroxy-, meta-ethoxy-, meta-isopropoxy-, ortho-hydroxy-, and ortho-ethoxy-derivatives were synthesized from the corresponding acetylated phenyl-substituted phenylalanines, each of which had been prepared by the α-chymotrypsin-catalyzed hydrolysis of the corresponding racemic methyl esters. (The five exceptions just mentioned were synthesized from the corresponding methoxy derivatives.) The hydrolyses of the racemic esters in the presence of α-chymotrypsin were continued until the apparent pH of the solution was nearly constant, indicating the rate of production of acid had almost ceased. With one exception, this point corresponded to almost complete hydrolysis of one optical isomer (93% or more). Isolation of the methyl esters remaining and methyl esterification of the isolated acids from each of the hydrolyses produced esters with identical melting points and optical rotations equal in magnitude but opposite in sign (see the second Appendix). The one exception was the para-chloro derivative, which was hydrolyzed to only 79%. The choice of enzyme and substrate concentrations apparently were such that the combination led to a very slow enzyme-catalyzed hydrolysis. The reaction was not allowed to proceed to completion for fear that the uncatalyzed hydrolysis would become of such relative importance that contamination of the free acid with its optical antipode would become significant. In this case, the unhydrolyzed ester had a lower melting point and a rotation whose magnitude was consistent with

the apparent concentration of the contaminating isomer calculated from the apparent extent of hydrolysis.

These results indicate the stereospecific hydrolyses of the acetylated phenyl-substituted-DL-phenylalanine methyl esters in the presence of α-chymotrypsin. They do not establish the configuration of the two products. There are two known cases in which the acylated D amino acid ester is hydrolyzed in the presence of α-chymotrypsin faster than the L antipode (17, 19, 22).

The ortho- and meta-hydroxy derivatives were synthesized from the corresponding methyl ethers which had been resolved by the enzyme. The ortho-ethoxy-, meta-ethoxy-, and meta-isopropoxy-derivatives were in turn synthesized from the hydroxy compounds by the same methods used for the preparation of the para-alkoxy-phenylalanine derivatives (29, 46). Treatment of the acetylated methyl ester remaining after hydrolysis of N-acetyl-meta-methoxy-DL-phenylalanine methyl ester in the presence of α-chymotrypsin with refluxing hydriodic acid gave an amino acid whose rotation was the same in magnitude and sign with that reported for meta-hydroxy-D-phenylalanine (47). Furthermore, the difference in magnitude of the rotation of this amino acid in aqueous and aqueous acid solution was consistent with the D configurational assignment (by the Clough-Lutz-Jirgensen rule) (48, 49). Similar treatment of the unhydrolyzed material from the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-ortho-methoxy-DL-phenylalanine methyl ester gave an acid whose rotation in water compared to its rotation in aqueous acid demonstrated its configuration as the D isomer (49). Thus, all the isomeric hydroxy and alkoxy derivatives in Table I are of the L configuration.

Bennett and Niemann reported the papain-catalyzed synthesis of phenylhydrazides from the isomeric N-acetyl-DL-fluorophenylalanines (50). Papain selectively catalyzed the synthesis of only one of the two enantiomeric N-acetyl-fluorophenylalanine phenylhydrazides. Only the phenylhydrazide of N-acetyl-L-phenylalanine was synthesized in the presence of papain, so it seemed likely that the L antipodes of the N-acetyl-fluorophenylalanines were also substrates of this enzyme-catalyzed reaction. The rotations of the isomeric acetylated fluorophenylalanines isolated from the α-chymotrypsin-catalyzed hydrolyses of their racemic methyl esters were equal in magnitude but opposite in sign to the rotations of the corresponding acetylated fluorophenylalanines isolated from the papain-catalyzed phenylhydrazide syntheses. Thus, they are enantiomorphs of one another. Since the stereochemical specificity of both enzymes is for the L isomer in the case of the corresponding non-fluoro-substituted phenylalanine derivatives (1, 17, 50), the conclusion that this specificity is preserved for the fluoro-phenylalanines for both enzymes seems a valid one. Further support for the assignment of the L antipodal specificity to both these enzymes toward the para-fluoro phenylalanine derivatives is provided by the finding that hydrolysis of chloroacetyl-para-fluoro-DL-phenylalanine by bovine carboxypeptidase yields the unacylated amino acid whose rotation is the same as that assigned the L configuration by Bennett and Niemann (50, 51). The antipodal specificity of carboxypeptidase is usually for the L antipode (51).

There remain seven compounds in Table I whose stereochemical configurations have not been investigated. All of these

compounds incorporate a substituted benzyl side chain in their structure. Their ko values are of a magnitude that places them in the class of "good" substrates of α-chymotrypsin (17). It therefore is reasonable to assume that the usual antipodal specificity of α-chymotrypsin for L acetylated amino acid esters (1, 5, 17, 30) was maintained in the hydrolyses of the parent racemic esters catalyzed by this enzyme.

Derivatives of N-Acetyl-L-phenylalanine Methyl Ester as Substrates of α-Chymotrypsin

The data obtained from the α-chymotrypsin-catalyzed hydrolyses of N-acetyl-L-phenylalanine methyl ester and several of its phenyl-substituted derivatives are presented in Table I. The three columns of data list ko, Ko, and the ratio (ko/Ko) for each of these substrates.

a) The ratio (ko/Ko) for derivatives of N-acetyl-L-phenylalanine methyl ester

The ratio (ko/Ko) for any substrate of α-chymotrypsin is a composite measure of the stability of the productive enzyme-substrate complexes and the ability of the complexes to decompose to products (6, 17). These ratios therefore allow quantitative comparisons of the overall efficiency with which α-chymotrypsin catalyzes the hydrolyses of several substrates. The third column of Table I indicates the wide range of catalytic activity exhibited by α-chymotrypsin in the hydrolysis of even this limited series of compounds - the largest ratio is 17,000 times the smallest.

b) Possible interpretations of the constants ko and Ko

Several theories have been proposed which could account for the variation in binding specificity and efficiency of catalysis with substrate structure in the α-chymotrypsin-catalyzed hydrolyses of the derivatives of N-acetyl-L-phenylalanine methyl ester. Each of them will be considered in turn.

It has been proposed that the act of binding of the substrate to the enzyme invariably increases the susceptibility of the substrate to reaction in proportion to the stability of the enzyme-substrate intermediate (52). A theory incorporating this idea - the "rack" mechanism - explains the enzymatic activation of a substrate towards reactivity by postulating that the binding of the substrate to the enzyme activates the bond which is broken by distorting or stretching it (52, 53). This theory predicts that the efficiency of enzymatic catalysis is relatively high when the binding is relatively good. An examination of the data presented in Table I reveals that in this series of substrates, relatively low Ko values do not generally insure high ko values and all high ko values are not associated with low Ko values. Therefore, it is concluded that the "rack" mechanism (52, 53) or similar strain theories of enzyme action (54) cannot be used to discuss the data in Table I.

A theory proposed by Koshland accounts for the specificity and efficiency of enzyme-catalyzed reactions in terms of the effect binding of a substrate to an enzyme has on the enzyme (55, 56). An important assumption of Koshland's "induced fit" theory is that the formation of an enzyme-substrate or enzyme-inhibitor complex causes considerable conformational changes in the enzyme. A distinguishing feature of the enzyme-substrate interaction is that

the substrate induces a conformation in the three-dimensional structure of the active site of the enzyme which brings the catalytic groups into the proper orientation for enzymatic activity (56). Optical rotatory dispersion studies of α-chymotrypsin and irreversibly acylated chymotrypsins at pH 4.0 have been interpreted as indicating conformational changes in this enzyme upon reaction with a substrate (57, 58). Similar optical rotatory dispersion studies with α-chymotrypsin and a substrate, N-acetyl-L-tyrosine ethyl ester, and with the enzyme and an inhibitor, hydrocinnamic acid, as a function of pH were interpreted as indicating no conformational changes in the enzyme at pH's near the optimum value of 7.9 (59). However, conformational changes were proposed above and below this pH (59). The objection that the binding of chromophoric groups to the enzyme may cause changes in the optical rotatory dispersion curves of a protein independent of any conformational changes in the protein structure (54), casts doubt on both these interpretations. Fluorescence studies of α-chymotrypsin in the presence of a few of its substrates and reversible inhibitors at pH 8.0 led Sturtevant (60) to conclude that these compounds cause conformational changes in the enzyme which do not represent formation of reaction intermediates and do not appear to be directly related to the enzyme-catalyzed reaction. Because there is no evidence in favor of substrate-induced conformational changes in the enzyme structure favorable to enzymatic activity at the optimum pH, this hypothesis will not be invoked to explain the results presented in Table I.

The formation of acyl-enzyme intermediates in all hydrolyses catalyzed by α-chymotrypsin was proposed (61) soon after the

discovery that acetyl-chymotrypsin is formed during the α-chymotrypsin-catalyzed hydrolysis of para-nitrophenyl acetate (62). The kinetic scheme for an α-chymotrypsin-catalyzed reaction involving an acyl-enzyme intermediate is the following

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

The form of the steady-state rate law for this scheme is the same as the Michaelis-Menten-Henri rate equation (62) (equation 1 in the Introduction), with $ko = k_2k_3/(k_2 + k_3)$ and $Ko = k_3(k_{-1} + k_2)/k_1(k_2 + k_3)$. If the rate of deacylation (k_3) is less than or of the same order of magnitude as the acylation rate (k_2), the acylation and deacylation steps will affect the magnitude of both ko and Ko (63, 64).

At least two authors (1, 54) have questioned the validity of the assumption of acyl-enzyme intermediates in <u>all</u> hydrolyses catalyzed by α-chymotrypsin. Because of this doubt and because the specificity theory based on acyl-chymotrypsin intermediates (64) does not permit analysis of the limited data available in Table I, it will not be used to interpret the ko and Ko values for the derivatives of N-acetyl-<u>L</u>-phenylalanine methyl ester.

A theory which is applicable to the interpretation of the constants in Table I is that of Hein and Niemann (17). Two general principles used by these authors will be adopted for the discussion of the constants for the derivatives of N-acetyl-<u>L</u>-phenylalanine

methyl ester. First, high ko values in α-chymotrypsin-catalyzed hydrolyses of mutually comparable substrates (e. g., acetylated L-α-amino acid methyl esters) are interpreted as an indication of optimal positioning of the hydrolyzable function in the ρ_3 locus. This interpretation emphasizes the entropy effects of the catalytic action of the enzyme on the substrate bound to it. Second, the Ko constant is considered an equilibrium dissociation constant. There are several lines of evidence which indicate that this is the case (1, 6, 65, 66). If Ko is an equilibrium dissociation constant, any change in experimental conditions or in substrate structure which changes Ko need not change ko, and vice versa. The data presented in Table I are consistent with this idea. Furthermore, as an equilibrium dissociation constant, Ko is a measure of stability of the complex (or complexes, if the substrate can bind to the enzyme in several mutually competitive modes) relative to the free enzyme and the free substrate in solution. As such, it is a measure of how well the substrate fits in the active site of the enzyme.

Hein and Niemann concluded that the interaction of N-acetyl-L-phenylalanine methyl ester with α-chymotrypsin involves predominantly one binding mode (17). This binding mode maintains acetylamino-ρ_1, carbomethoxy-ρ_3, and side chain-ρ_2 interactions. Calculations performed recently by Dr. Carole Hamilton (67) indicate that the contributions of alternative binding modes to the overall binding energy of N-acetyl-L-phenylalanine methyl ester to α-chymotrypsin are slight. Since all the compounds in Table I are derivatives of N-acetyl-L-phenylalanine methyl ester and incorporate bulky side chains in their structure, it is reasonable to assume that the frequency of occurrence of alternative binding modes to the

predominating acetylamino-ρ_1, carbomethoxy-ρ_3, and side chain-ρ_2 mode is slight in all cases. The discussion of the ko and Ko values may therefore involve a consideration of only the side chain-ρ_2 interactions of the substrates listed in Table I with α-chymotrypsin and their perturbations on the other binding interactions.

c) A model for the ρ_2 locus

Information is at hand which permits visualization of the gross dimensions and geometry of the ρ_2 locus of the active site of α-chymotrypsin. Before the present investigation was completed, it was known that N-acetyl-L-phenylalanine methyl ester (23, 24) and N-acetyl-L-tryptophan methyl ester (68) are substrates of α-chymotrypsin-catalyzed hydrolyses. The constants for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester are ko = (51 ± 7) sec^{-1} and Ko = (0.12 ± 0.03) x 10^{-3}M (68). Although it is believed that the α-chymotrypsin-catalyzed hydrolyses of tryptophan derivatives involve a higher frequency of non-productive binding modes than do the corresponding phenylalanine compounds (6, 17, 67), the β-indolylmethyl side chain of N-acetyl-L-tryptophan methyl ester must be bound at ρ_2 for this substrate to be hydrolyzed at all. In fact, the side chain-ρ_2 interaction of N-acetyl-L-tryptophan methyl ester must allow a carbomethoxy-ρ_3 interaction which is at least as favorable to catalytic hydrolysis as the carbomethoxy-ρ_3 interaction of N-acetyl-L-phenylalanine methyl ester. The higher frequency of non-productive binding modes for the tryptophan derivative should lower its ko value more than do the non-productive binding modes of the phenylalanine compound, yet the ko of N-acetyl-L-tryptophan methyl ester is only slightly lower

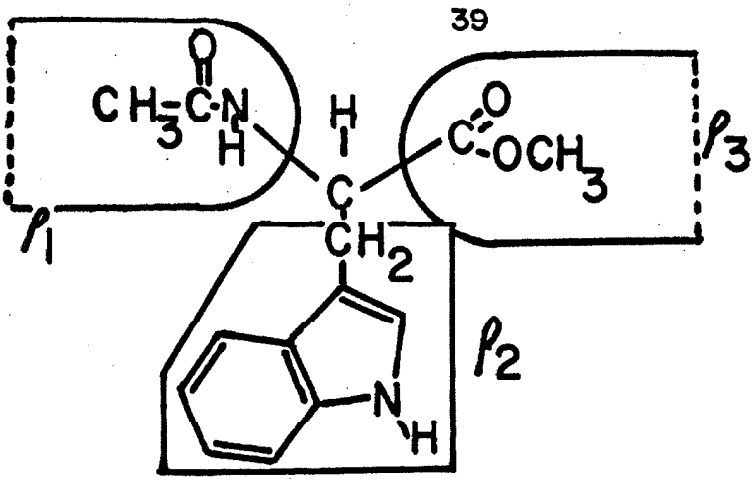
than the ko of N-acetyl-L-phenylalanine methyl ester. Both these compounds have identical R_1 groups (acetylamino), and it can be assumed that the geometry of the R_1-ρ_1 interactions are identical. If the geometry of the carbomethoxy-ρ_3 interactions are the same for the two compounds, the geometry of the side chain-ρ_2 interactions must be nearly the same. This argument suggests the gross size and shape of the ρ_2 locus, which is illustrated in Figure 2.

It is expected that the discussion of the ko and Ko values of the compounds will permit a refinement of this simplified picture.

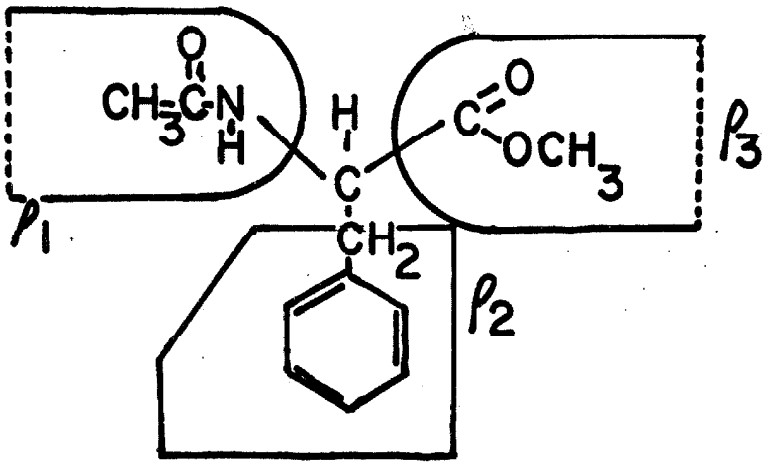
d) The ko values of derivatives of N-acetyl-L-phenylalanine methyl ester

Because it is assumed that predominantly productive enzyme-substrate complexes are formed, the ko values may be interpreted as direct measurements of the rate of decomposition of the productive complexes to products (1, 6, 17). Variations in the magnitude of ko may then be attributed largely to differences in the free energy of activation needed to promote each of the various enzyme-substrate intermediates to the transition state leading to products.

There are two ways by which the substituents on the benzyl side chain could affect the free energy of activation. If the ρ_2 locus is such that the side chains of all the phenylalanine derivatives fit in ρ_2 in essentially the same manner, the positioning of the carbomethoxy groups of each of them with respect to the catalytic groups in ρ_3 should be essentially the same. The variations in ko could then be explained in terms of a transmission of electronic effects of the substituents through the phenyl ring and the two saturated



N-Acetyl-L-tryptophan methyl ester bound to the active site in a productive mode.



N-Acetyl-L-phenylalanine methyl ester bound to the active site in a productive mode.

Figure 2. Shape and size of ρ_2.

carbons to the carbomethoxy groups. On the other hand, the effect of the substituents may be to alter the interaction between the enzyme and the substrate such that the positioning of the carbomethoxy group with respect to the catalytic groups in ρ_3 varies from one derivative to another.

Substitution on the phenyl ring of β-phenylpropionic acid has a small, but measureable, effect on the ionization of its carboxyl group and on the hydrolysis of its ethyl esters (69). However, in the compounds being discussed, the presence of the α-acetylamino group undoubtedly has more influence than the substituted phenyl ring on the reactivity of the carbomethoxy group. The pKa's of acetic acid, β-phenylpropionic acid, and acetylglycine in aqueous solutions at 25°C are 4.76, 4.66, and 3.67, respectively (70). The much larger ability of the acetylamino group to stabilize the negatively charged carboxyl and the small effect of substituents on the ionization of β-phenylpropionic acid and the hydrolysis of the corresponding ethyl ester suggest that the carbomethoxy groups of all the substrates listed in Table I should be equally susceptible to hydrolysis whether hydrolysis is catalyzed or not. If the substituents do influence the susceptibility of the carbomethoxy groups to hydrolysis, a Hammett correlation might be followed, since all the substrates are subject to the same catalyst under the same experimental conditions. A comparison of some of the ko values for the substrates in Table I with the appropriate Hammett σ constants (71) demonstrates that transmission of substituent effects to the carbomethoxy group is not an explanation for the observed variation in ko values (see Table VI). Thus, there is no evidence which invalidates the conclusion that the enthalpies of

TABLE VI: Hammett Sigma Constants and ko Constants for α-Chymotrypsin-catalyzed Hydrolyses of Derivatives of N-Acetyl-L-phenylalanine Methyl Ester.

Substituent	Hammett Sigma (71)	ko(sec^{-1})	log ko
H	0	67	1.83
meta-methyl	-0.07	77	1.89
meta-methoxy	+0.08	250	2.40
meta-hydroxy	+0.10	245	2.40
meta-fluoro	+0.34	120	2.09
meta-chloro	+0.37	130	2.11
meta-nitro	+0.78	380	2.58
para-hydroxy	-0.18	117	2.07
para-amino	-0.17	118	2.07
para-methoxy	-0.11	0.49	-0.31
para-methyl	-0.09	27	1.43
para-fluoro	+0.06	64	1.81
para-chloro	+0.24	13	1.11
para-nitro	+0.78	18	1.26

activation of the intermediates formed between these substrates and α-chymotrypsin are essentially identical. It is concluded that the variations in ko with changes in the substrate structure may be explained largely in terms of the entropy of activation of the enzyme-substrate intermediates. This variation in the entropy of activation is in turn considered to be a variation in the access of the carbomethoxy group to the catalytic groups of ρ_3 in the enzyme-substrate complex. Since the only variable feature of the substrate structure is the substituent on the benzyl side chain, the side chain-ρ_2 interactions of the various derivatives must be the cause of the variations in the ko values.

The importance of the entropy of activation in terms of the optimal positioning of the hydrolyzable group in ρ_3 depends on the number of catalytic groups needed for reactivity, the spatial relationship among them needed for catalytic activity, and the restriction on the number of spatial relationships which can be assumed by them. None of these factors has been established experimentally. It is generally recognized, however, that the achievement of a favorable geometry between the catalytic groups and the group being hydrolyzed is essential in α-chymotrypsin-catalyzed reactions (1, 17, 63, 65, 72).

Structure-reactivity correlations of the substrates listed in Table I are facilitated by grouping the substrates according to the magnitudes of their ko values. There are three distinct groups: substrates with ko values of 200 sec^{-1} to 380 sec^{-1}, substrates with ko's of 10 sec^{-1} to 30 sec^{-1}, and substrates with ko's of less than 1 sec^{-1}. A factor of ten has been chosen arbitrarily as an indication of a definite decrease in reactivity. Several substrates have ko values falling between these groups.

Four substrates- the meta-nitro, meta-hydroxy, meta-methoxy, and meta-isopropoxy derivatives- belong to the first group. The 3,5-dimethoxy derivative may also, but the uncertainty in its ko value is so large that such a classification is tenuous. Apparently a necessary, but not sufficient, requirement for high ko values is substitution at the position meta to the methylene of the benzyl group.

The second group includes the ortho-chloro, ortho-hydroxy, meta-ethoxy, para-chloro, para-methyl, para-nitro and para-ethoxy derivatives.

The para-methoxy and para-isopropoxy derivatives have the lowest ko values of all the compounds listed in Table I. Both these compounds are hydrolyzed in the presence of α-chymotrypsin with ko's of less than 1 sec^{-1}.

The conclusion that the variation in ko with changes in the substituent on the benzyl side chain is a result of variations in fit of the side chain in ρ_2 permits a refinement of the description of the ρ_2 locus (see Figure 2) by a correlation of the nature of the side chains with the magnitude of the appropriate ko's. The first group of substrates that will be considered is the group containing the derivatives of N-acetyl-L-phenylalanine methyl ester with the highest ko values, i.e., the meta-nitro, meta-hydroxy, meta-methoxy and meta-isopropoxy derivatives.

The size of the substituent groups in these four derivatives varies considerably (see Table VII). Furthermore, the maximum van der Waals radii of the methyl and chloro groups are nearly the same as that of the hydroxy group, yet the ko of the meta-hydroxy derivative is twice that of the meta-chloro and over three times

TABLE VII: Sizes of the Substituent Groups

Substituent	d[a]	r[b]	References
Hydrogen	2.2	1.2	73
Fluorine	2.7	1.4	73, 74
Chlorine	3.6	1.8	73, 75
Methyl	3.5	2.0	73
Hydroxyl	3.1	1.7	73, 76
Amino	3.2	2.0	73, 77
Nitro	3.5	2.5	73, 78, 79
Methoxyl	4.1	2.7	73, 80
Ethoxyl	4.6	2.7	73, 80
Isopropoxyl	4.6	2.9	73, 80

a) In angstrom units. The definition of d is the distance, along the bond of the substituent to the phenyl ring, from the center of phenyl carbon adjacent to the substituent to the "outside" of the substituent.

b) In angstrom units, The definition of r is the maximum van der Waals radius of the substituent from the center of the atom attached to the phenyl ring.

that of the meta-methyl derivatives. The size of a meta-substituent may contribute to its effectiveness in increasing ko, but it is not the dominant factor.

The meta-nitro-, meta-hydroxy-, and meta-alkoxy-benzyl groups all have dipole moments of varying magnitudes, but in the same general sense (i. e., directed away from the phenyl ring and toward the substituent) (81, 82). The magnitude of the dipole moment of the meta substituents along the direction of the bond from the phenyl ring to the substituent decreases in the following order: nitro > halo > hydroxy or alkoxy (see Table VIII). However, the magnitude of ko values decrease in the order: meta-nitro > meta-hydroxy or meta-alkoxy > meta-halo. It is therefore concluded that an interaction of the dipole at the meta position with an ion or dipole at ρ_2 of the active site is not a sufficient explanation for the observed order of reactivity.

The basicities of nitro-benzene, phenol, and alkyl ethers of phenol are weak, but measurable (83, 84). The oxygens of the phenolic hydroxyl and of alkyl phenolic ethers can accept a proton to form either an intermolecular or an intramolecular hydrogen bond (85). Although the aromatic nitro group is much weaker as a base than the oxygens of either phenolic hydroxy or ether groups (83, 84, 86), it is considered capable of accepting a proton to form both intermolecular and intramolecular hydrogen bonds (86-88). The halogens of halo-substituted benzenes are immeasurably weak bases (86), but intramolecular hydrogen bonds have been proposed for compounds such as the ortho-halophenols (89, 90).

TABLE VIII: Dipole Moments of Toluene Derivatives (82).

Derivative	Maximum Dipole Moment[a]	Angle[b]	Dipole in the Substituent Bond Direction
para-Hydrogen	0.4	0	0.4
para-Fluoro	1.9	0	1.9
para-Chloro	2.0	0	2.0
para-Methyl	0.0	0	0.0
para-Nitro	4.4	0	4.4
para-Amino	1.2	130	0.8
para-Hydroxy	1.8	41	1.4
para-Methoxy	1.6	49	1.1
meta-Hydrogen	0.4	0	0.3
meta-Fluoro	1.7	49	1.7
meta-Chloro	1.8	49	1.8
meta-Methyl	0.4	298	-0.2
meta-Nitro	4.2	55	4.2
meta-Hydroxy	1.4	108	0.9
meta-Methoxy	1.2	87	1.0
ortho-Hydrogen	0.4	0	-0.3
ortho-Fluoro	1.3	104	1.2
ortho-Chloro	1.4	106	1.4
ortho-Hydroxy	1.2	181	0.6
ortho-Methoxy	0.9	170	0.6

a) In debyes.

b) In degrees, measured clockwise from an axis passing through the methyl group of toluene and the hydrogen or substituent para to the methyl.

In view of the ability of aromatic nitro, hydroxy, and alkoxy groups to function as acceptors of hydrogen bonds, it is proposed that a hydrogen bond is formed at the ρ_2 site by the meta-nitro, meta-hydroxy, meta-methoxy, and meta-isopropoxy derivatives with a proton donated by the enzyme. It is further proposed that this hydrogen bond restricts the side chain-ρ_2 interaction such that the carbomethoxy-ρ_3 interaction is optimal for catalysis. The meta-chloro and meta-fluoro derivatives should have less tendency to participate in hydrogen bond formation than the meta-nitro, meta-hydroxy, and meta-alkoxy derivatives. The resulting decrease in restriction on their side chain-ρ_2 interactions should lead to a decreased orientation of the carbomethoxy group in ρ_3 and lower ko values. The meta-methyl derivative should have no tendency to accept a hydrogen bond in its side chain-ρ_2 interaction and the close approximation of its ko value to that of N-acetyl-L-phenylalanine methyl ester is expected.

The conformation of the enzyme-substrate complex of the meta-ethoxy derivative is apparently different from those of the meta-nitro, meta-hydroxy, meta-methoxy, and meta-isopropoxy derivatives, since its ko value is less than one-tenth of the ko's of the latter compounds. This decrease in ko is not consistent with a mere diminished ability of the substrate to maintain an optimum positioning of the carbomethoxy group in ρ_3 because N-acetyl-L-phenylalanine methyl ester and its meta-methyl derivative, which cannot participate in hydrogen bonding at the ρ_2 locus, have ko values three to four times as large as the meta-ethoxy derivative. Therefore, the explanation of the diminished

reactivity of the meta-ethoxy derivative must involve an orientation of its carbomethoxy group in ρ_3 unfavorable to reactivity.

Maximum resonance stabilization of phenol and alkyl phenyl ethers requires coplanarity of the oxygen-hydrogen and the oxygen-alkyl bonds with the phenyl ring (91). The maximum wave lengths and the corresponding extinction coefficients of the ultraviolet spectra of phenol, anisole, phenetole, and isopropyl phenyl ether in cyclohexane are closely similar, indicating that this coplanarity is achieved for all of them (91, 92). The basicities of these compounds in sulfuric acid are also consistent with this structural feature (83). In order to avoid steric repulsions with the phenyl ring, the branching methyl groups on the ethyl and isopropyl ethers may be forced into positions which partially shield the ether oxygens from a hydrogen bond donor. This shielding effect or a steric barrier at the site of the hydrogen bond donor could prevent the meta-ethoxy and meta-isopropoxy derivatives from achieving the side chain-ρ_2 interaction accessible to the meta-nitro, meta-methoxy, and meta-hydroxy derivatives. The alternative binding mode or modes for the side chain of the meta-ethoxy derivative in ρ_2 apparently prevent its carbomethoxy group from achieving a positioning in ρ_3 like that available to the meta-hydroxy, meta-methoxy, and meta-nitro derivatives. However, the meta-isopropoxy derivative has a ko value almost identical to those of the meta-hydroxy and meta-methoxy derivatives, indicating that the positioning of its carbomethoxy group in ρ_3 must be near optimal. Yet the shielding of the ether oxygen of the isopropoxy derivative from a potential hydrogen bond donor and the possible repulsion by some steric barrier must be worse than for the

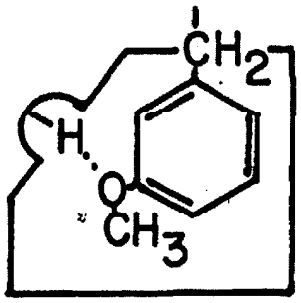
ethoxy derivative. There does not seem to be an obvious explanation for this apparent contradiction and the ko value for the meta-ethoxy derivative is considered anomalous.

For the present, the representation of the ρ_2 locus given in Figure 2 must be modified to include the postulated proton-donating group accessible to substituents which are located meta to the methylene of a phenylalanine derivative's side chain. This modification is represented in Figure 3.

The lowest ko values are observed for the para-methoxy and para-isopropoxy derivatives. The size and location of the substituents on the benzyl side chains of these compounds suggests that a steric barrier is met in the interaction of the side chains with ρ_2. It is of interest to consider the other derivatives of N-acetyl-L-phenylalanine methyl ester substituted at the para position in order to determine the nature of their interactions with ρ_2.

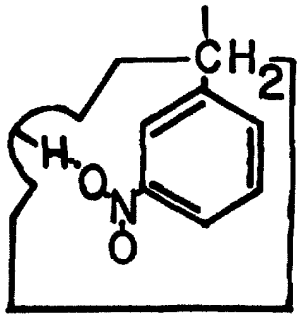
Among the para-substituted derivatives, the para-hydroxy and para-amino derivatives have the highest ko values. The methyl and chloro groups are about the same size as the amino and hydroxy groups (see Table VII), yet the para-methyl and para-chloro derivatives have ko values only about one-seventh or less than the para-amino and para-hydroxy derivatives. A simple steric requirement for a substituent in the para position is therefore ruled out.

An interaction of the dipole of a substituent at the para position of the benzyl side chain with an ion or dipole in ρ_2 is ruled out by comparing the dipole moments of toluene derivatives (see Table VIII) with the ko values of the corresponding derivatives of N-acetyl-L-phenylalanine methyl ester substituted at the para



H = hydrogen bond donating group

Restriction of interaction of m-methoxybenzyl side chain at ρ_2 by hydrogen bonding.



Restriction of interaction of m-nitrobenzyl side chain at ρ_2 by hydrogen bonding.

Figure 3. Modified model of the ρ_2 locus.

position of the benzyl side chain. There is no consistent correlation between the two quantities.

Among the para-substituted derivatives, both the amino and hydroxy groups can accept a proton to form a hydrogen bond, but a nitro group can also (85, 86). The para-nitro derivative has a ko value only about one-seventh that of the para-amino and para-hydroxy derivatives. This rules out the possibility of a hydrogen bond formed between a proton donated by a group at the ρ_2 locus and a substituent at the para position of a benzyl side chain.

Both the para-amino and para-hydroxy derivatives could form a hydrogen bond by donation of a proton to the ρ_2 locus and are the only para-substituted derivatives which can (93). It is proposed that a hydrogen bond accepting group is located at a site in ρ_2 accessible to para-substituted derivatives of N-acetyl-L-phenylalanine methyl ester and that interactions of the side chains of the para-amino and para-hydroxy derivatives with this group lead to orientations of their carbomethoxy groups in ρ_3 favorable to reactivity.

The lengths of the side chains of the para-chloro, para-methyl, and para-nitro derivatives along an axis passing through the methylene and substituent groups are nearly the same (see Table VII). If the hydrogen bond accepting group in ρ_2 is near to, or associated with, a steric barrier, the carbomethoxy groups of these three derivatives should be disoriented in ρ_3 to approximately the same extent. The ko values of all three of them are approximately equal.

The side chains of the para-alkoxy derivatives are even longer than those of the para-chloro, para-methyl, and para-nitro derivatives. It is expected that the ko values of the para-alkoxy derivatives should be even lower than those of the latter. The ko values for the para-methoxy and para-isopropoxy derivatives are the lowest observed for any of the phenylalanine derivatives. These low values are consistent with the interpretation offered for the ko values of the para-methyl, para-chloro, and para-nitro derivatives.

The side chain-ρ_2 interaction of the para-ethoxy derivative must be different from those of the other para-alkoxy derivatives, since its ko value is at least twenty times the ko values of the para-methoxy and para-isopropoxy derivatives. An alternative interaction which avoids the postulated steric barrier in ρ_2 must be available to the para-ethoxy benzyl side chain. This interaction may also be available to the meta-ethoxy derivative, since their ko values are very similar. If similar side chain-ρ_2 interactions are available to these two derivatives, it is reasonable to suppose that it involves some location in the ρ_2 locus between the postulated hydrogen bond donating and hydrogen bond accepting sites. Possibly this interaction occurs in the space in ρ_2 available for the β-indolylmethyl side chain of N-acetyl-L-tryptophan methyl ester. It is unclear why this interaction should be available to the side chain of the para-ethoxy derivative and not to the para-methoxy and para-isopropoxy compounds. The para-ethoxy and meta-ethoxy derivatives must still be considered anomalies.

The para-fluoro derivative is sterically equivalent to the parent compound, N-acetyl-L-phenylalanine methyl ester, because

the fluorine and hydrogen atoms are nearly the same size. It is therefore expected that the carbomethoxy groups of the two compounds should be equally well positioned in ρ_3 and their ko values nearly identical. The observed ko values of the two compounds are essentially the same.

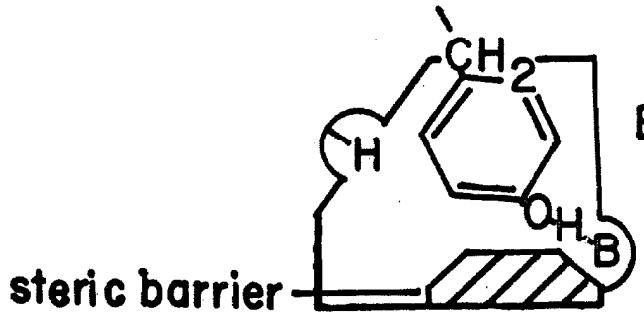
The representation of the ρ_2 locus given in Figure 3 may now be modified to include the proposed proton-accepting group accessible to derivatives of N-acetyl-L-phenylalanine methyl ester substituted at the para position of the benzyl side chain and the proposed steric barrier. The modified picture is given in Figure 4.

The remaining derivatives of N-acetyl-L-phenylalanine methyl ester are all substituted at the position ortho to the methylene of the benzyl side chain.

Comparisons of the dipole moments of the toluene derivatives with the ko values of the ortho-substituted derivatives of N-acetyl-L-phenylalanine methyl ester demonstrate that there is no ion-dipole or dipole-dipole interaction of these compounds with ρ_2.

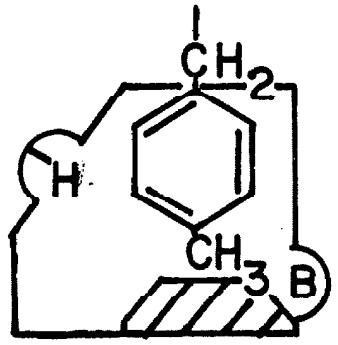
The ortho-fluoro derivative is sterically equivalent to the para-fluoro derivative and to N-acetyl-L-phenylalanine methyl ester. The close similarity observed between the ko's of the three compounds is expected. The meta-fluoro derivative has a ko value about twice that for the other three, possibly because of an improved orientation of its carbomethoxy group in ρ_3 caused by the formation of a hydrogen bond with a proton donated by the enzyme.

The ko values of the ortho-chloro, ortho-hydroxy, ortho-methoxy, and ortho-ethoxy derivatives are all less than that of



B = hydrogen bond accepting group

Restriction of interaction of p-hydroxybenzyl side chain at ρ_2 by hydrogen bonding.



Restriction of interaction of p-methylbenzyl side chain at ρ_2 by encounter of a steric barrier.

Figure 4. Further modification of the model for the ρ_2 locus.

N-acetyl-L-phenylalanine methyl ester. Furthermore, the values decrease with increasing size of the substituent. The chloro and hydroxy groups have nearly the same ko values and nearly the same maximum van der Waals radii. The ortho-methoxy and ortho-ethoxy derivatives have lower ko values than the ortho-chloro and ortho-hydroxy groups, in accordance with the increase in size of the substituents.

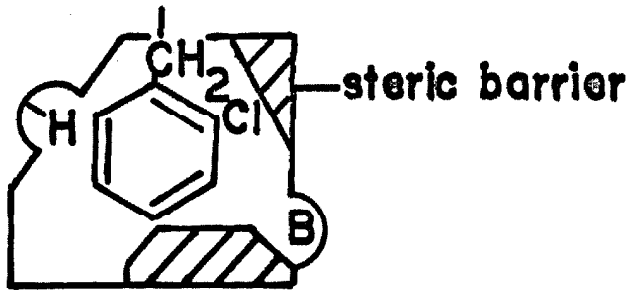
Abrash and Niemann found that N-acetyl-2, 6-dimethyl-DL-tyrosine methyl ester has very low activity as a substrate of α-chymotrypsin (94). They concluded that steric repulsions between the β-methylene hydrogens and the two ortho-methyl groups prevent a side chain-ρ_2 interaction similar to that achieved in the interaction of N-acetyl-L-phenylalanine methyl ester with α-chymotrypsin (94). A steric shielding of the reactive carbonyl by the ortho-methyl groups like that proposed for the β-branching methyl groups of N-acetyl-L-valine methyl ester (95, 96) was considered unlikely (94).

Steric repulsions of the methylene hydrogens with the hydrogen of the ortho-hydroxy and the alkyl part of the ortho-alkoxy groups should be avoided by directing the hydrogen or alkyl groups away from the methylene and toward the meta hydrogen. Therefore, the type of steric repulsions which arise in Abrash and Niemann's substrate should be unimportant in the ortho-hydroxy and ortho-alkoxy derivatives. The chlorine atom has a slightly smaller van der Waals radius than the methyl group, so that it also may avoid steric repulsions with the methylene hydrogens. It will therefore be assumed that the ortho-substituted benzyl groups can form the same sort of interactions with the hydrophobic

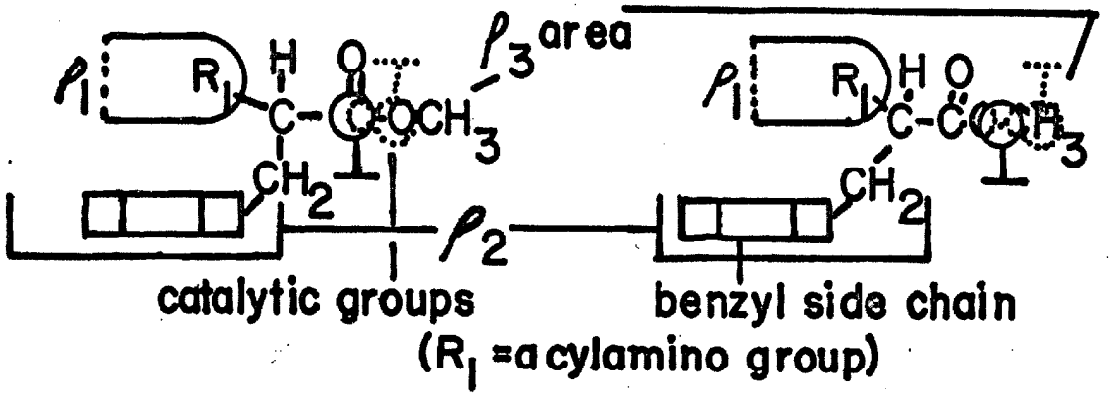
ρ_2 (17) as the benzyl group of N-acetyl-L-phenylalanine methyl ester.

It is proposed that the explanation for the behaviour of the ortho-substituted derivatives of N-acetyl-L-phenylalanine methyl ester as substrates of α-chymotrypsin involves the encounter of the ortho substituents with a steric block in the ρ_2 locus. A steric constraint in ρ_2 at the region corresponding to the space between the methylene of the benzyl group and the position ortho to it could prevent the side chain from attaining a position in the ρ_2 locus which orients the carbomethoxy group in ρ_3 for maximum reactivity. An alternative explanation is that the ρ_2 and ρ_3 loci of the active site partially overlap so that an ortho substituent can interfere with the attack of the catalytic groups in ρ_3 on the carbomethoxy group. The first explanation is preferred, since Abrash and Niemann concluded that no overlapping of the two loci occurs in the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-2, 6-dimethyl-DL-tyrosine methyl ester (94).

The final modification of the representation of the ρ_2 locus may now be presented. As shown in Figure 5, the dimensions of ρ_2 are such that groups as small as a methyl or a chloro group at the ortho or para positions can lower ko by preventing the proper positioning of the carbomethoxy group of the substrate with respect to the catalytic groups in ρ_3. It should be remembered that this model is based on the rate with which the various enzyme-substrate complexes decompose to products and therefore represents the size and shape of ρ_2 after the side chain of a substrate has combined with it.



Restriction of interaction of o-chlorobenzyl side chain at ρ_2 by encounter of a steric barrier.



Effect of side chain-ρ_2 interaction on positioning of hydrolyzable group in ρ_3. (Optimal positioning is represented on the left.)

Figure 5. Final modification of the model for the ρ_2 locus (top) and illustration of orientation of the hydrolyzable group in ρ_3 by the side chain-ρ_2 interaction.

e) The Ko values of derivatives of N-acetyl-L-phenylalanine methyl ester

The assumption has been made that the Ko constants may be considered to be equilibrium dissociation constants of the enzyme-substrate complex. Each dissociation constant is a measure of the difference in the sum of the ground state free energies of the enzyme and substrate in solution and the ground state free energy of the enzyme-substrate complex in solution. The ground state free energy of the uncomplexed enzyme in solution must have been the same for all the kinetic determinations used to obtain the data summarized in Table I because the experimental conditions (i.e., ionic strength, temperature, etc.) were the same for all the determinations. In comparing the Ko constants for the various substrates, the variation in Ko may be attributed to the variation in the difference between the ground state free energy of the substrate in solution and the ground state free energy of its complex with α-chymotrypsin.

The variation in ground state free energy of the enzyme-substrate complex will depend primarily on the favorability of the side chain-ρ_2 interaction, since the binding interactions at ρ_1 and ρ_3 should be the same for all the substrates in Table I. The possibility exists, however, that the conformation of a side chain-ρ_2 interaction can disturb the acetylamino-ρ_1 and carbomethoxy-ρ_3 interactions. Such an occurrence should be reflected in the magnitude of ko as well as Ko.

The active site of α-chymotrypsin is expected to possess a certain amount of mobility (1). There must be definite limits to this mobility (or flexibility); otherwise,no specificity would be

observed (1). In this discussion of Ko values, the active site, in general, and the ρ_2 locus, in particular, will be considered to be essentially rigid. The ρ_2 locus should consequently have the same size and structural characteristics as the representation of ρ_2 given in Figure 5.

The ground state free energies of the derivatives of N-acetyl-L-phenylalanine methyl ester in aqueous solutions should be a function of the solvation of these compounds by water. A qualitative indication of this solvation is obtained by comparing the solubilities of various benzene and toluene derivatives substituted analogously to the derivatives of N-acetyl-L-phenylalanine methyl ester. This is only a very crude approximation, but it should be adequate for the largely qualitative comparisons which will be made among the Ko values for the various derivatives of N-acetyl-L-phenylalanine methyl ester.

The solubilities of several benzene and toluene derivatives in water are listed in Table IX. Assuming no unusual temperature dependence of the solubilities of these compounds in water and that, to a first approximation, the ground state free energies of the derivatives of N-acetyl-L-phenylalanine methyl ester in water correspond to the solubilities of the aromatic compounds in water, the following approximate ordering of the ground state free energies of the derivatives of N-acetyl-L-phenylalanine methyl ester is obtained: nitro, chloro > alkoxy, fluoro, methyl, unsubstituted > hydroxy, amino. (Since the comparison is only qualitative, no distinction is made among the different positions of the substituents.)

If electronic differences between the side chains of the isomeric fluoro derivatives of N-acetyl-L-phenylalanine methyl

TABLE IX: Solubilities of Benzene and Toluene Derivatives in Water

Compound	Solubility[a]	Temperature[b]	Reference
Benzene	10	22	97
Fluorobenzene	16	30	97
Chlorobenzene	4.3	30	97
Anisole	about 10	25	98
Toluene	5.1	16	97
meta-Nitrotoluene	3.6	30	97
para-Nitrotoluene	3.2	30	97
ortho-Cresol	230	30	99
meta-Cresol	46	30	99
para-Cresol	170	30	99
para-Toluidine	70	21	97

a) In units of 10^{-3}M.

b) In ^{o}C.

ester and the benzyl group of the parent compound are ignored, it is expected that all four compounds should have essentially the same Ko values. The three isomeric fluoro derivatives should be as well solvated as the parent compound in water and, because the size of the fluorine atom is about the same as that of the hydrogen atom, the ground state free energies of their complexes with α-chymotrypsin should be about the same as that of the parent compound. In fact,the Ko values of these four substrates of α-chymotrypsin agree to within twenty-five percent.

The chloro- and methyl-substituted derivatives are nearly equivalent sterically, since the van der Waals radii of the two groups are almost equal. It is therefore expected that the ground state free energies of the enzyme-substrate complexes of the para-chloro and the para-methyl derivatives should be about the same. The meta-methyl and meta-chloro derivatives should also form complexes with α-chymotrypsin that have about the same ground state free energies. (The electronic differences between the chlorobenzyl and the methylbenzyl side chains are ignored.) The difference in Ko values may be attributed to the solvation of the substrates in solution. Since chlorobenzene is less soluble in water than toluene, the chloro-substituted derivatives should be less well solvated than the methyl-substituted derivatives. The ortho-chloro derivative has a larger Ko than its isomers. This observation is consistent with the steric obstruction postulated for the interaction of the ortho-substituted derivatives with α-chymotrypsin, which should raise the level of the ground state free energy of the complex of the enzyme with the ortho-chloro compound above the ground state free energy

level of each of the complexes of the enzyme with the meta-chloro and para-chloro compounds.

The similarity in magnitude of each of the Ko values for the meta- and para-methyl derivatives to the Ko of the unsubstituted parent compound suggests that the increase in size of the side chain in these two cases does not raise the ground state free energy level of the enzyme-substrate complex. Apparently the area available in ρ_2 for interaction with a substrate side chain is, on the average, large enough to accommodate a meta- or para-methylbenzyl side chain. This conclusion is consistent with the conclusion reached earlier based on consideration of the ko values.

It is expected that the ground state free energies of the meta-hydroxy, para-hydroxy, and para-amino derivatives in aqueous solution should each be lower than the ground state free energy of the unsubstituted parent compound. Steric hindrance to solvation by the acetylamino and carbomethoxy groups and the possibility of an intramolecular hydrogen bond between the amido hydrogen and the ortho-hydroxyl might raise the ground state free energy of the ortho-hydroxy substrate in aqueous solution above the ground state free energy of the meta-hydroxy substrate or the para-hydroxy substrate in aqueous solution.

The maximum van der Waals radii of the hydroxy, the amino, the chloro and the methyl groups are all approximately equal (see Table VII). If it is assumed that the only criterion for assigning a relative ground state free energy to an enzyme-substrate complex of a derivative of N-acetyl-L-phenylalanine methyl ester is the ability of the side chain to fit the ρ_2 locus (the other R-ρ interactions being essentially identical), the ground

state free energies of the complexes of α-chymotrypsin with the substrates substituted in the meta and para positions with these four groups should all be approximately the same. Since it was concluded that the ρ_2 locus could bind a meta-methylbenzyl or para-methylbenzyl side chain as easily as an unsubstituted benzyl side chain, the ground state free energies of the complexes of these derivatives with α-chymotrypsin should each be almost equal to the ground state free energy of the complex of the unsubstituted compound with α-chymotrypsin. The ground state free energies of the complexes of α-chymotrypsin with the ortho-hydroxy substrate and with the ortho-chloro substrate should be approximately equal to each other, by the criterion of fit.

Comparisons of the differences between the qualitative ground state free energies for the enzyme-substrate complex and the substrate in aqueous solution among the several methyl, chloro, hydroxy and amino derivatives lead to several predictions regarding the Ko's of these compounds. The Ko of the ortho-hydroxy derivative should be larger than the Ko of the ortho-chloro derivative, because the ortho-hydroxy derivative is the better solvated of the two in aqueous solution. The observed Ko of the ortho-hydroxy compound is about four times the Ko of the ortho-chloro compound. The meta-hydroxy substrate has a Ko thirty times the Ko of the meta-methyl substrate. This qualitative difference is also expected on the basis of the estimated solvation energies of the two compounds in water. Because it should be better solvated in water than the para-methyl compound, the para-amino compound should have the larger Ko of the two. The observed Ko of the para-amino substrate is three times the Ko of the para-methyl substrate. The observed

Ko of the para-hydroxy derivative is smaller than the Ko of the para-methyl derivative, contrary to what would have been predicted on the basis of the qualitative criteria of solvation of the substrates in solution and of fitting of the side chains in the ρ_2 locus. Furthermore, application of these criteria would lead to the prediction that the Ko's of the para-amino and the para-hydroxy derivatives should be the same, contrary to experimental observations. It was proposed in the discussion of the ko values that a group is present in the ρ_2 locus which can accept a proton from an amino or hydroxy group substituted at the para position of the benzyl side chain of N-acetyl-L-phenylalanine methyl ester to form a hydrogen bond. A phenolic hydroxyl has a stronger tendency to donate a proton to form a hydrogen bond than the amino group of an aromatic amine (85, 92). The increased stability of the hydrogen bond to the ρ_2 locus for the para-hydroxy derivative relative to the para-amino derivative could result in a lower Ko for the para-hydroxy compound than for the para-amino compound.

It was decided earlier that the ground state free energies of the nitro derivatives in aqueous solutions should be approximately equal to the ground state free energies of the chloro derivatives in aqueous solutions. The maximum van der Waals radius of the nitro group is about one and one half times the maximum van der Waals radius of the chloro group. There is a possibility that the meta-nitrobenzyl side chain and the para-nitrobenzyl side chain of the meta-nitro and para-nitro derivatives, respectively, do not fit into the ρ_2 locus as easily as do the corresponding chloro derivatives. It is also possible that some property related to the electronic nature of the nitrobenzyl side chains is involved in the process of binding of these substrates to the enzyme. The crude

approximations regarding the ground state solvation of the derivatives of N-acetyl-L-phenylalanine methyl ester in aqueous solutions may not be applicable to comparing the nitro compounds to the other derivatives of N-acetyl-L-phenylalanine methyl ester. In the absence of a better means for estimating the ground state free energies of these substrates in aqueous solutions, no firmer conclusions can be drawn.

The alkoxy derivatives of N-acetyl-L-phenylalanine methyl ester should all have approximately the same ground state free energies in aqueous solution as the unsubstituted parent compound. The magnitudes of the observed Ko's of the alkoxy derivatives must then be determined by the ground state free energies of the complexes of each of the alkoxy compounds with the enzyme.

Generally, the ortho-alkoxy and para-alkoxy derivatives have lower Ko values than the meta-alkoxy derivatives. The ko values of the ortho-alkoxy and para-alkoxy compounds are also lower than the ko values of the meta-alkoxy derivatives. This behavior is reminiscent of the type of behavior associated with substrates of α-chymotrypsin for which a predominance of non-productive binding modes is invoked (17). However, it is difficult to visualize binding modes for these substrates with α-chymotrypsin which does not involve a side chain-ρ_2 interaction. Possibly, the binding of these substrates to α-chymotrypsin involves, as the predominant binding mode, an R_2-ρ_2 interaction, with no other R-ρ interactions. Whatever the binding interaction is, it is apparently the result of the increased bulk of the alkoxy substituent. This conclusion is consistent with the proposed steric barriers in ρ_2 which correspond to the ortho and para positions of the benzyl side chains.

The relatively high Ko values of the meta-alkoxy derivatives and the 3, 5-dimethoxy derivative suggest that the large bulk of these substrates requires an expansion or stretching of ρ_2 in order to accomodate the large side chains. Apparently the average size of ρ_2 is not "wide" enough to easily accomodate these side chains. It would be of interest to evaluate the substrate activities of the meta-ethyl, meta-n-propyl and meta-isobutyl derivatives of N-acetyl-L-phenylalanine methyl ester with α-chymotrypsin. These compounds are sterically equivalent to the meta-alkoxy derivatives but cannot accept a proton to form a hydrogen bond. The ability of the meta-alkoxy derivatives to accept a proton to form a hydrogen bond may make it possible for them to combine with the enzyme to form predominantly productive complexes in spite of their large size.

Analogs of N-Acetyl-L-tyrosine Methyl Ester as Substrates of α-Chymotrypsin

Hein and Niemann (17) proposed that when an acylated α-amino acid derivative is hydrolyzed under the catalytic influence of α-chymotrypsin, the role of the acylamino-ρ_1 interaction is to aid in the positioning of the hydrolyzable function with respect to the catalytic groups in ρ_3 so that efficient catalysis occurs. They postulated that replacement of the acylamino group by a less polar group or a group with a modified geometry could decrease the ko value by as much as a factor of 10^4 (17). This postulate was based, in part, on the observation that replacement of the amido nitrogen of N-acetyl-DL-phenylalanine by a methylene group to give methyl DL-α-benzyllevulinate results in a decrease in ko of a factor of 10^3(6).

Table III reveals how the replacement of the acetylamino group of N-acetyl-L-tyrosine methyl ester by a hydrogen atom and by an N-methylacetylamino group changes the kinetic constants for the α-chymotrypsin-catalyzed hydrolysis of a typical substrate. Replacement of the acetylamino group by a hydrogen atom changes ko by a factor of approximately 3×10^{-3} and increases Ko by a factor of approximately three. A decrease in ko by a factor of about 2×10^{-4} and an increase in Ko by a factor of more than 25 accompanies N-methylation.

The assumptions and principles which will be used to interpret these results are essentially those used in the discussion of the kinetic constants of the α-chymotrypsin-catalyzed hydrolysis of the derivatives of N-acetyl-L-phenylalanine methyl ester.

a) The ko constants of N-acetyl-L-tyrosine methyl ester and two analogs

The modification of the R_1 (acetylamino) group of N-acetyl-L-tyrosine methyl ester could change the ko of its α-chymotrypsin-catalyzed hydrolysis in two ways. The susceptibility of the carbomethoxy group to hydrolysis (catalyzed or uncatalyzed) might be changed by a substituent effect. If such a substituent effect is unimportant, the modification of the R_1 group could change ko by changing the positioning of the carbomethoxy group relative to the catalytic groups in ρ_3 in the enzyme-substrate complex, as proposed by Hein and Niemann (17). A combination of these two effects is also possible.

If it is assumed that the substituent effects of the R_2 (para-hydroxybenzyl, in this case) and the R_1 (hydrogen, acetylamino or

N-methylacetylamino) groups on the susceptibility of the carbomethoxy group to hydrolysis are additive, the substituent effects of the various R_1 groups may be evaluated. Taft has proposed a scheme for evaluating substituent effects on the reactions of carboxylic acids and their derivatives (100, 101). A ρ^* value of 1.00 is assumed for the base-catalyzed hydrolyses of aliphatic esters (100, 101). The methyl groups on the acyl and alkyl groups of the general ester RCOOR are each assigned a σ^* value of 0.00. The ρ^* and σ^* values in this scheme are then applied to Hammett-type free energy relationships. The ρ^* value for ionization of aliphatic acids is 1.72 (100). From the ionization constants of acetic acid and acetylglycine (4.76 and 3.67, respectively (70)), a σ^* value of +0.63 may be calculated for the acetylaminomethyl group. The base-catalyzed hydrolyses of N-acetylglycine and N-methyl-N-acetylglycine methyl esters proceed with second-order rate constants of 2.7 M^{-1} sec^{-1} and 1.1 M^{-1} sec^{-1}, respectively, in aqueous solutions 0.5 M in sodium chloride, at pH 7.9 and 25° (18a, b). The derived σ^* of the N-methyl-N-acetylaminomethyl group is +0.24. There is no ρ^* value which is applicable to the α-chymotrypsin-catalyzed hydrolyses of methyl esters because of the difficulty in comparing the characteristics of the enzyme-substrate complexes of various substrates with α-chymotrypsin (1, 6, 17). However, the differences in the magnitudes of the calculated σ^* constants shows that these changes in the R_1 group could alter the susceptibility of the carbomethoxy group to hydrolysis by a substituent effect. If it is assumed that, except for the R_1-ρ_1 interaction, the conformations of the complexes of these three substrates with α-chymotrypsin are identical, that the

frequency of non-productive binding modes (17) is the same for all three, and that the substituent effect of the para-hydroxybenzyl group is constant and additive to the substituent effect of the R_1 group in each case, the order of the ko constants should be: methyl para-hydroxyhydrocinnamate < N-acetyl-N-methyl-L-tyrosine methyl ester < N-acetyl-L-tyrosine methyl ester. It is observed experimentally that the ko of methyl para-hydroxyhydrocinnamate is almost ten times that of N-acetyl-N-methyl-L-tyrosine methyl ester. It is not possible to decide whether the change in R_1 affects the ko by a combination of a substituent effect with a change in the interaction of the substrate with the enzyme or by a change in the substrate-enzyme interaction alone. It is clear, however, that a substituent effect cannot by itself explain these data.

The change in the R_1 group could affect the enzyme-substrate interaction in two ways. The frequency of non-productive modes of binding may change as a result of the change in R_1. On the other hand, the character of the productive mode could change while the frequency of non-productive modes remains essentially constant.

The likelihood of an increase in frequency of non-productive modes of binding of these substrates to α-chymotrypsin with these changes in the R_1 group does not seem very great. The replacement of an acetylamino group by a hydrogen should not increase the frequency of an R_1-ρ_2 interaction, because of the small size of the hydrogen atom. Branching of a side chain at a position two carbons removed from the carboxyl derivative causes the binding constant Ko to increase (17, 95, 96). It is, therefore, not expected

that an N-methylacetylamino group can compete more effectively with a para-hydroxybenzyl group for ρ_2 than can an acetylamino group. Because of its small size and because carbonyl groups are apparently necessary for effective interactions at ρ_3 (6, 17), the hydrogen atom should have no more tendency to interact at ρ_3 than an acetylamino group. Branching of the alcoholic moiety of ester substrates of α-chymotrypsin leads to increases in Ko (17, 95, 96). An N-methylacetylamino group should, for that reason, not be better bound at ρ_3 than an acetylamino group. The frequency of the interactions of the carbomethoxy and para-hydroxybenzyl groups with ρ_1 should not be changed by the change in R_1. The changes in ko with changes in R_1 must, therefore, be the result of a modified productive binding mode.

Modification of the productive binding mode by modification of the R_1 group must involve a disorientation of the carbomethoxy group with respect to the catalytic groups in ρ_3. The acetylamino-ρ_1 interaction presumably restricts the positioning of the carbomethoxy group in ρ_3 to a configuration with respect to the catalytic groups which is favorable to reactivity (17). The α-hydrogen atom of methyl para-hydroxyhydrocinnamate, because it is too small or because it lacks the proper functional group, apparently cannot interact with ρ_1 to force the carbomethoxy group of the substrate into the reactive configuration with respect to the catalytic groups in ρ_3. The R_1 group of N-acetyl-N-methyl-L-tyrosine methyl ester either may not interact with ρ_1 in the same manner as an acetylamino group (because of the loss of the amido hydrogen (5, 29)) to achieve a reactive configuration between the carbomethoxy group and the catalytic groups in ρ_3, or the increased bulk of the

R_1 group may sterically block the attack of the catalytic groups on the ester function (29). The Ko value of the N-methyl compound could aid in making a decision on this point.

b) The Ko constants of N-acetyl-L-tyrosine methyl ester and its analogs

The Ko constants of these substrates are assumed to be equilibrium dissociation constants, like the Ko constants of the derivatives of N-acetyl-L-phenylalanine methyl ester. Neither methyl para-hydroxyhydrocinnamate nor N-acetyl-N-methyl-L-tyrosine methyl ester should be so well solvated in aqueous solutions as N-acetyl-L-tyrosine methyl ester. Of the three compounds, only N-acetyl-L-tyrosine methyl ester incorporates an acetylamino group, which should be well solvated by hydrogen bonding in water (102), in its structure. Consequently, the ground state free energy of N-acetyl-L-tyrosine methyl ester in aqueous solutions should be lower than the ground state free energy of either of the other two compounds in aqueous solutions. If the ground state free energies of the complexes of the enzyme with all three substrates were equal, the Ko values of methyl para-hydroxyhydrocinnamate and N-acetyl-N-methyl-L-tyrosine methyl ester would each be smaller than the Ko of N-acetyl-L-tyrosine methyl ester. Since the Ko of N-acetyl-L-tyrosine methyl ester is actually smaller than the Ko for either of the other two compounds, they must form complexes with the enzyme which have higher ground state free energy levels than does the complex of α-chymotrypsin with N-acetyl-L-tyrosine methyl ester.

The predominant binding mode for the interaction of methyl para-hydroxyhydrocinnamate with α-chymotrypsin should involve para-hydroxybenzyl-ρ_2 and carbomethoxy-ρ_3 interactions. The Ko of methyl para-hydroxyhydrocinnamate is approximately three times the Ko of N-acetyl-L-tyrosine methyl ester. It can therefore be concluded that the acylamino-ρ_1 interaction contributes significantly to the energy of the binding interaction of an acylated α-amino acid derivative to α-chymotrypsin.

N-Methylation of the acetylamino group leads to an even greater increase in the Ko value than does replacement of the acetylamino group by a hydrogen atom. The predominant binding mode of methyl para-hydroxyhydrocinnamate with α-chymotrypsin involves carbomethoxy-ρ_3 and para-hydroxybenzyl-ρ_2 interactions, yet methyl para-hydroxyhydrocinnamate has a Ko only about one-eighth that of the N-methyl derivative. Apparently N-methylation of the acetylamino function prevents its effective interaction with the ρ_1 locus of the enzyme and so distorts the binding mode of the substrate to α-chymotrypsin that effective side chain-ρ_2 and carbomethoxy-ρ_3 interactions are not achieved. The methylation of the acetylamino nitrogen may prevent its effective interaction at ρ_1 in at least two ways. The size of the acylamino function is greatly increased by methylation and steric repulsions may prevent its binding at ρ_1. Alternatively, there may be a requirement for a group which can donate a hydrogen bond to ρ_1 for a successful interaction at ρ_1.

c) Conclusion

The proposal of Hein and Niemann (17), that an acylamino-ρ_1 is necessary for optimum reactivity of an acylated α-amino acid

derivative as a substrate of α-chymotrypsin, has been confirmed. In addition, it has been found that the acylamino-ρ_1 interaction makes a significant contribution to the binding of the substrate to α-chymotrypsin.

Derivatives of Methyl Hippurate as Substrates of α-Chymotrypsin

The kinetic constants for the α-chymotrypsin-catalyzed hydrolyses of several derivatives of methyl hippurate substituted at the phenyl ring of the benzoyl moiety are presented in Table IV. The kinetic constants for the derivatives of methyl hippurate are compared to the constants for the appropriate derivatives of N-acetyl-L-phenylalanine methyl ester in Tables X and XI.

The ko value of the α-chymotrypsin-catalyzed hydrolyses of N-acetyl-L-phenylalanine methyl ester can be enhanced by substitution at the benzyl side chain of the substrate. Substitution on the benzamido group of methyl hippurate never increases the ko of hydrolysis of that substrate in the presence of α-chymotrypsin.

The Ko value of N-acetyl-L-phenylalanine methyl ester is sensitive to changes in the substituent on the benzyl side chain. Substituents on the benzamido group of methyl hippurate affect the Ko value in only two cases and the change in Ko does not parallel the analogous change in the phenylalanine series.

a) Interpretation of the kinetic constants for the α-chymotrypsin-catalyzed hydrolyses of derivatives of methyl hippurate

The side chain-ρ_2 interactions of derivatives of acylated glycine, L-alanine and D-alanine with α-chymotrypsin are weak

TABLE X. The ko Values for Derivatives of Methyl Hippurate and of N-Acetyl-L-phenylalanine Methyl Ester[a]

Substituent	ko (Hippurate deriv.)	ko (Phenylalanine deriv.)
None	0.22	67 ± 2
meta-Methoxy	0.23 ± 0.04	250 ± 60
meta-Ethoxy	0.004 ± 0.001	19 ± 1
para-Amino	0.027 ± 0.001	120 ± 10
para-Methoxy[b]	< 0.001	0.49 ± 0.03
para-Ethoxy	0.002 ± 0.001	10 ± 1
para-Isopropoxy[b]	< 0.001	0.012 ± 0.005

a) In units of sec^{-1}. See Tables I and IV.

b) No measurable substrate activity at 1.4×10^{-4}M α-chymotrypsin.

TABLE XI. The Ko Values for Derivatives of Methyl Hippurate and N-Acetyl-L-phenylalanine Methyl Ester [a]

Substituent	Ko (Hippurate deriv.)	Ko (Phenylalanine deriv.)
None	5.3	0.6 ± 0.1
meta-Methoxy	3 ± 1	5 ± 2
meta-Ethoxy	4 ± 2	22 ± 2
para-Amino	3 ± 1	3.0 ± 0.5
para-Methoxy[b]	0.6 ± 0.1	2.6 ± 0.4
para-Ethoxy[b]	4 ± 1	12 ± 2
para-Isopropoxy[b]	16 ± 10	1.6 ± 0.4

a) In units of 10^{-3}M. See Tables I and IV.

b) Evaluated as K_I. See Tables IV and V.

because of the small size of the side chains (1, 17, 18a, b, 22). As the size of the acyl function on the α-amino group approaches that of the benzyl side chain of N-acetyl-L-phenylalanine methyl ester, the frequency of the acylamino-ρ_2 interaction increases and can, in principle, predominate over the acylamino-ρ_1 interaction. For glycine derivatives, two binding modes, both of them potentially productive, should predominate (18a, b, 22). One of these binding modes incorporates the acylamino-ρ_1 interaction and the other incorporates the acylamino-ρ_2 interaction. Both of them incorporate the carbomethoxy-ρ_3 interaction, and place a hydrogen atom in the ρ_H locus.

An extensive study of the behaviour of acylated glycine, L-alanine, and D-alanine methyl esters as substrates of α-chymotrypsin was made by Rapp (22). From the results with aromatic and heteroaromatic acyl groups, it was concluded that the predominant binding mode of these three classes of substrates of α-chymotrypsin involved an acylamino-ρ_2, carbomethoxy-ρ_3 interaction (22). This binding mode is expected to be productive for the acylated D-alanine methyl esters and non-productive for the corresponding L enantiomers (17, 21, 22). Those aromatic or heteroaromatic acyl groups which increase ko for the acylated D-alanine methyl esters also increase ko for the acylated glycine methyl esters, but decrease ko for the acylated L-alanine methyl esters (22). It was concluded that the predominant and productive binding mode for both the acylated glycine and D-alanine methyl esters is the acylamino-ρ_2, carbomethoxy-ρ_3 interaction (22).

It will be assumed in the discussion of the kinetic constants for the α-chymotrypsin-catalyzed hydrolyses of the derivatives of

methyl hippurate listed in Table IV that the predominant and productive binding mode is the one incorporating the acylamino-ρ_1, carbomethoxy-ρ_3 mode. The Ko constants will be considered equilibrium dissociation constants.

b) The ko constants of derivatives of methyl hippurate

If it is assumed that the predominant and productive binding mode for the acylated glycine methyl esters listed in Tables IV and X is the one incorporating an acylamino-ρ_2 interaction, it might be expected that the ko values of derivatives of methyl hippurate substituted at the phenyl ring of the benzoyl moiety should parallel the ko values of analogously substituted derivatives of N-acetyl-L-phenylalanine methyl ester. However, the size and geometry of the groups linking the phenyl ring to the carbomethoxy group are different in the two cases. The steric barrier encountered by ortho-substituted derivatives of N-acetyl-L-phenylalanine methyl ester in ρ_2 may be encountered by the amide function of the substituted benzoylamino group. Improvement of the positioning of the carbomethoxy group with respect to the catalytic groups in ρ_3 by interaction of the substituted benzoylamino group in ρ_2 would then not be possible.

Apparently, there is an unfavorable steric interaction of the amide function of the benzoylamino group in ρ_2, since substitution on the phenyl of the benzoyl group never enhances ko (see Table X). However, the ko values of the substituted methyl hippurates parallel the ko values of the analogously substituted derivatives of N-acetyl-L-phenylalanine methyl ester. In both

series of derivatives, the meta-methoxy derivative has the highest ko value, the para-amino derivative has the next highest ko value, etc. This behaviour is consistent with the conclusion of Rapp (22) that the predominant and productive binding mode in the α-chymotrypsin-catalyzed hydrolyses of methyl esters of glycine acylated with an aromatic or heteroaromatic group is the acylamino-ρ_2, carbomethoxy-ρ_3 mode.

c) The Ko and K_I constants of derivatives of methyl hippurate

The inhibition constants of competitive inhibitors of α-chymotrypsin are true equilibrium dissociation constants (98). The binding constants of substrates of α-chymotrypsin may also be considered equilibrium dissociation constants (1, 5, 6, 17). The Ko or K_I value of each derivative of methyl hippurate may be considered a measure of the difference in the ground state free energies of the substrate in solution and of its complex with α-chymotrypsin.

All the methyl hippurate derivatives, with the possible exception of the para-amino derivative, should be approximately equally well solvated in water. Therefore, all of them (except the para-amino derivative) should have approximately the same ground state free energies in aqueous solution. The para-amino substrate may have a lower ground state free energy in water than the other derivatives of methyl hippurate. With the one exception, the Ko and K_I values can be considered a measure of the effectiveness of the binding of the substituted benzoylamino group in ρ_2.

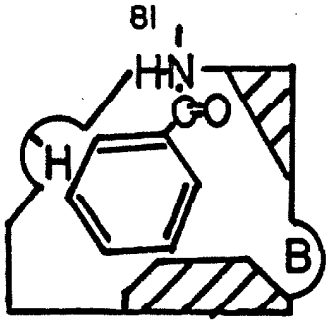
The Ko constants of the meta-methoxy-, meta-ethoxy-, and para-ethoxy hippuric acid methyl esters are similar to one another and close to the Ko of methyl hippurate. All these compounds are substrates of α-chymotrypsin, although the ko constants of the ethoxy derivatives are extremely low. Methyl hippurate and its meta-methoxy derivative are undoubtedly bound to α-chymotrypsin in similar modes since the kinetic constants of the two compounds are similar. Apparently the dominating effect on the ground state free energies of the complexes of both compounds with α-chymotrypsin is the steric effect of the amido function. This proposed steric effect must also dominate the free energy of the ground state of each of the ethoxy derivatives with α-chymotrypsin, since each has a Ko close to the Ko of methyl hippurate.

Methyl para-aminohippurate has a Ko essentially equal in magnitude to the Ko of methyl hippurate. The ground state free energy of the complex of α-chymotrypsin with the para-amino derivative must then be lower than the ground state free energy of the complex of methyl hippurate with α-chymotrypsin, since methyl para-aminohippurate is assumed to be the better solvated of the two substrates in water. The donation of a proton by the para-amino substrate to a group in ρ_2 may explain this difference in the ground state free energies of the two enzyme-substrate complexes.

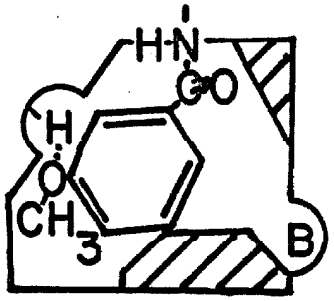
The para-isopropoxy derivative has a much higher K_I value than the other alkoxy derivatives of methyl hippurate. Apparently the increase in size of the acylamino group prevents both productive and effective combination with ρ_2.

The low K_I value for the para-methoxy derivative is unexpected. It is difficult to rationalize this low value in terms of the model proposed for ρ_2 on the basis of the kinetic constants for the α-chymotrypsin-catalyzed hydrolysis of derivatives of N-acetyl-L-phenylalanine methyl ester and the discussion of the Ko and K_I constants for the other derivatives of methyl hippurate.

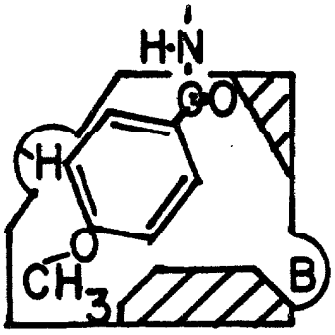
A pictorial representation of the interaction of the acylamino group of the derivatives of methyl hippurate with the ρ_2 locus is presented in Figure 6.

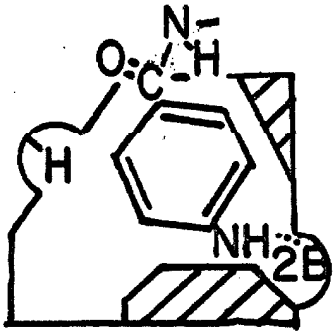


Restriction of interaction of benzamido group of methyl hippurate at ρ_2 by encounter of asteric barrier.



Interaction of m-methoxybenzamido group at ρ_2.





Interactions of p-methoxybenzamido and p-aminobenzamido groups at ρ_2.

Figure 6. Interactions of the acylamino groups of derivatives of methyl hippurate at ρ_2.

EXPERIMENTAL

Syntheses

All melting points were corrected by a calibration curve constructed by Dr. Alan Platt. Analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan, and by Elek Microanalytical Laboratories, Los Angeles, California.

N-Acetyl-L-phenylalanine Methyl Ester

L-Phenylalanine (16.5 g., 0.10 mole) was esterified with methanol (100 ml.) and thionyl chloride (14.3 g., 0.12 mole) to give the crude methyl ester hydrochloride (103). The crude product was suspended in ethyl acetate (100 ml.) and acetylated with acetic anhydride (15 ml., 21 g., 0.20 mole) in the presence of potassium carbonate (55 g., 0.40 mole) in water (100 ml.) (23). The yield of crude material was 14 g. (63%). This crude product was crystallized four times from isopropyl ether to give N-acetyl-L-phenylalanine methyl ester, m.p. 90-91° and $[\alpha]_D^{21}$ = + (19.4 ± 0.3)° (C, 2.04% in methanol). Jones and Niemann report m.p. 90°, $[\alpha]_D^{20}$ = + (17.8 ± 0.2)° (C, 2% in methanol) (23). Huang et al. report m.p. 90-91°, $[\alpha]_D^{25}$ = + 19° (C, 2% in methanol) (104).

N-Acetyl-L-tyrosine Methyl Ester

Esterification of L-tyrosine (22 g., 0.12 mole) with methanol (70 ml.) and thionyl chloride (17 g., 0.14 mole) in the

usual manner yielded the crude methyl ester hydrochloride (103). This product was dissolved in the minimum amount of hot water and the free amino acid ester was obtained by addition of 1 N aqueous sodium hydroxide. The free ester was acetylated with acetic anhydride (46 ml., 64 g., 0.63 mole) to give 25 g. (95%) of crude N-acetyl-L-tyrosine methyl ester, m.p. 134-136° (105). The crude material was crystallized five times from ethyl acetate to give the product, m.p. 134-135°, $[\alpha]_D^{22}$ = + (30 ± 1)° (C, 0.55% in methanol). Values reported in the literature are: m.p. 136-137° (uncorrected), $[\alpha]_D^{20}$ = + 29.7 (C, 0.4% in methanol) (106).

α-N-Acetyl-α-N-methyl-L-tyrosine Methyl Ester

This compound was prepared by Hubele (29). It was recrystallized twice from ethyl acetate-ligroin to give a product with m.p. 134°, $[\alpha]_D^{25}$ = - (81.5 ± 0.5)° (C, 0.96% in pyridine).

Analysis:	calculated	C: 62.1	H: 6.8	N: 5.6
$C_{13}H_{17}NO_4$ (251)	found	C: 62.2	H: 6.8	N: 5.5

N-Acetyl-O-methyl-L-tyrosine Methyl Ester

This compound was synthesized by Hubele (29). It was recrystallized twice from toluene to give a product, m.p. 104-105°, $[\alpha]_D^{25}$ = + (25.3 ± 1.4)° (C, 0.36% in methanol).

Analysis:	calculated	C: 62.1	H: 6.8	N: 5.6
$C_{13}H_{17}NO_4$ (251)	found	C: 62.3	H: 6.8	N: 5.6

N-Acetyl-O-ethyl-L-tyrosine Methyl Ester

This product was also synthesized by Hubele (29). It was recrystallized twice from toluene to give the acetylated amino acid ester, m. p. 117-118.5°, $[\alpha]_D^{25} = + (29.3 \pm 0.4)^\circ$ (C, 1.06% in methanol).

Analysis:	calculated	C: 63.4	H: 7.2	N: 5.3
$C_{14}H_{19}NO_4$ (265)	found	C: 63.5	H: 7.1	N: 5.4

N-Acetyl-O-isopropyl-L-tyrosine Methyl Ester

This compound, also synthesized by Hubele (29), gave after two crystallizations from a diethyl ether-ligroin mixture, m. p. 58-59°, $[\alpha]_D^{25} = + (34.0 \pm 0.4)^\circ$ (C, 2.04% in methanol).

Analysis:	calculated	C: 64.5	H: 5.0	N: 7.6
$C_{15}H_{21}NO_4$ (284)	found	C: 64.6	H: 5.0	N: 7.7

p-Nitro-L-phenylalanine

L-Phenylalanine (12.5 g., 0.075 mole) was dissolved in concentrated (95%) sulfuric acid (40 g., 0.39 mole) at 30-40° (maintained by cooling with an ice bath). While this solution was stirred, concentrated (15 M) nitric acid (5 ml., 0.075 mole) was added, dropwise and with cooling, at a rate such that the temperature was maintained at 20-30°C. After all the concentrated nitric acid had been added (over a 15 minute period), another 0.5 ml. concentrated nitric acid was added. There was no further increase

in temperature, indicating completion of the nitration. The reaction solution was stirred for 15 minutes and poured into 500 ml. water contained in an ice bath. The strongly acidic, yellow solution was brought to a pH of about 8 with concentrated aqueous ammonium hydroxide. The resulting white product was filtered and washed with cold water. The filtrate was concentrated under vacuum to approximately 150 ml., the pH was readjusted with concentrated aqueous ammonia hydroxide to pH 8 and a second crop was collected. The combined crude yield was 12.1 g. (76%). After two crystallizations from water, the product gave $[\alpha]_D^{26} = + (9.9 \pm 0.6)^o$ (C, 1.66% in N aqueous hydrochloric acid). Bergel and Stock report $[\alpha]_D^{25} = + (9.8 \pm 0.3)^o$ (C, 1.77% in N aqueous hydrochloric acid) (47).

N-Acetyl-p-nitro-L-phenylalanine

p-Nitro-L-phenylalanine (1.5 g., 0.0071 mole) was dissolved in 10 ml. of aqueous 1 N sodium hydroxide and the solution was cooled to 5^o in an ice bath. This solution was treated with acetic anhydride (0.80 ml., 1.1 g., 0.11 mole) to effect acetylation by a Schotten-Baumann procedure similar to the one described by duVigneaud and Meyer for L-tyrosine (46). After the reaction mixture had been stored overnight at 10^o, it was acidified to give a white product which was filtered, washed with cold water and dried. The yield of crude acetylated p-nitro-L-phenylalanine was 1.2 g. (82%). The product had a m.p. of 195-197° and $[\alpha]_D^{25} = + (41 \pm 11)^o$ (C, 1.64% in ethanol). After two crystallizations from water the m.p. was 204-207° and $[\alpha]_D^{28} = + (48.5 \pm 0.7)^o$ (C,

1.65% in ethanol). Bergel and Stock report a m. p. of 206-209° and $[\alpha]_D^{25}$ = + (49.7 ± 1)° (C, 1.55% in ethanol) (107).

N-Acetyl-p-nitro-L-phenylalanine Methyl Ester

Esterification of p-nitro-L-phenylalanine (8.4 g., 0.040 mole) with methanol (80 ml.) and thionyl chloride (5.5 g., 0.046 mole) (103) gave the crude methyl ester hydrochloride. This product was washed with anhydrous diethyl ether and added, with stirring, to a mixture of sodium acetate (6.2 g., 0.076 mole) and acetic anhydride (33 ml., 46 g., 0.45 mole). The procedure followed was adapted from that for the acetylation of L-valine methyl ester hydrochloride reported by Applewhite et al. (108) and modified by Kurtz et al. (98). As in the modified acetylation procedure just mentioned, the acetic anhydride was destroyed by addition of water and application of heat. The resulting solution was evaporated to dryness and the residue was taken up in ethyl acetate. The ethyl acetate solution was washed with aqueous sodium bicarbonate (5%), then 0.5 N aqueous hydrochloric acid and finally a saturated aqueous sodium chloride solution. When the sodium chloride solution washings were neutral, the ethyl acetate solution was dried over anhydrous magnesium sulfate, treated with charcoal and filtered. The ethyl acetate was removed, leaving 7.9 g., (71%) of crude product, m. p. 118-120°. After four crystallizations from carbon tetrachloride, N-acetyl-p-nitro-L-phenylalanine methyl ester had m. p. 123-124° and $[\alpha]_D^{28}$ = + (22.0 ± 0.2)° (C, 2.09% in methanol).

Analysis:	calculated	C: 54.1	H: 5.3	N: 10.5
$C_{12}H_{14}N_2O_5$ (266)	found	C: 54.4	H: 5.3	N: 10.5

α-N-Acetyl-p-amino-L-phenylalanine Methyl Ester

A solution of N-acetyl-p-nitro-L-phenylalanine methyl ester (9.0 g., 0.034 mole) in 100 ml. of absolute ethanol was introduced into a Parr bottle and platinum oxide catalyst (0.10 g.) was added. The Parr bottle containing the solution was connected to a Parr hydrogenation apparatus and pressurized to 50 p. s. i. g. with hydrogen, after flushing the system several times with hydrogen. After the solution had been shaken under hydrogen pressure for 18 hours, the bottle was removed and the spent catalyst was filtered off. This reduction is adapted from the synthesis of ethyl p-aminobenzoate from ethyl p-nitrobenzoate described by Adams and Cohen (109).

Charcoal was added to the filtrate and then removed by filtration. Removal of the ethanol under reduced pressure gave a gummy residue which was taken up in an excess of diethyl ether. The ethereal solution was treated with charcoal, filtered and concentrated on the steam bath until white crystals began to appear in the solution. The crude product, obtained in a yield of 6.5 g. (81%), had a m. p. of 101-103°. Four additional crystallizations from diethyl ether gave a product having an m. p. 102-102.5° and $[\alpha]_D^{25}$ = + (28.7 ± 0.4)° (C, 1.27% in methanol).

Analysis:	calculated	C: 61.0	H: 6.8	N: 11.9
$C_{12}H_{16}N_2O_3$ (236)	found	C: 60.8	H: 6.9	N: 11.8

N-Acetyl-o-fluoro-DL-phenylalanine Methyl Ester

The starting material for this synthesis was N-acetyl-o-fluoro-DL-phenylalanine, m. p. 147-150°, prepared by E. L. Bennett (50).

The acetylated o-fluoro-DL-phenylalanine (9.0 g., 0.042 mole) was esterified using methanol (100 ml.) and thionyl chloride (7.1 g., 0.060 mole) (103). After removal of the methanol, the gummy residue was taken up in ethyl acetate and a small amount of water. The solution was washed with aqueous bicarbonate (5%), N aqueous hydrochloric acid, and saturated aqueous sodium chloride. The ethyl acetate solution was dried over magnesium sulfate, charcoal was added and the solution was filtered. Removal of the ethyl acetate gave 7.5 g. (76%) of N-acetyl-o-fluoro-DL-phenylalanine methyl ester, m.p. 79-80° after crystallizing from isopropyl ether.

Enzymatic Resolution of N-acetyl-o-fluoro-DL-phenylalanine Methyl Ester

A solution of N-acetyl-o-fluoro-DL-phenylalanine methyl ester (7.124 g., 0.02978 mole) in 75 ml. of methanol was added to 465 ml. water. To this was added a solution of α-chymotrypsin (107 mg.) in 10 ml. of water. The pH of the solution, determined by a Leeds and Northrup 7401 pH meter, was maintained between pH 7.0 and 8.0 by manual addition of 0.129 N aqueous sodium hydroxide. After 15 minutes, the pH of the solution remained essentially constant. The amount of base added was 111 ml., corresponding to a 96% hydrolysis of one optical isomer. The methanolic solution was extracted with approximately 700 ml. of ethyl acetate. The ethyl acetate solution was extracted with 5% aqueous sodium bicarbonate solution, 1 N aqueous hydrochloric acid and saturated aqueous sodium chloride. Finally, it was dried over magnesium sulfate, treated with charcoal and filtered.

The ethyl acetate solution was evaporated to dryness to give 2.5 g. (70%) of the crude D enantiomer. The product was crystallized four times from isopropyl ether to give N-acetyl-o-fluoro-D-phenylalanine methyl ester, m.p. 106-107°, $[\alpha]_D^{25}$ = - (10.1 ± 0.4)° (C, 1.27% in methanol).

N-Acetyl-o-fluoro-L-phenylalanine

The aqueous phase from the resolution described above was concentrated to approximately 20 ml. and acidified with concentrated aqueous hydrochloric acid. The yield of crude product was 1.5 g. (45%) of acetyl-o-fluoro-L-phenylalanine, m.p. 165-167°. After two crystallizations from water, the material had a m.p. of 171-172°, $[\alpha]_D^{28}$ = + (29 ± 1)° (C, 5.28% in ethanol). Bennett and Niemann give m.p. 168-170°, $[\alpha]_D^{25}$ = - 28.6° (C, 8% in ethanol) for N-acetyl-o-fluoro-D-phenylalanine (50).

N-Acetyl-o-fluoro-L-phenylalanine Methyl Ester

Esterification of N-acetyl-o-fluoro-L-phenylalanine (1.3 g., 0.0060 mole) by methanol (60 ml.) and thionyl chloride (1.8 g., 0.015 mole) was carried out similarly to the method of Brenner and Huber (103). The crude product, obtained in a yield of 0.9 g. (64%), was crystallized four times from isopropyl ether to give a product having a m.p. of 106-107°, $[\alpha]_D^{25}$ = + (10.5 ± 0.4)° (C, 1.26% in methanol).

Analysis:	calculated	C: 60.2	H: 5.9	N: 5.9
$C_{12}H_{14}NO_3F$ (239)	found	C: 60.5	H: 6.0	N: 6.0

N-Acetyl-m-fluoro-DL-phenylalanine Methyl Ester

This product was prepared in a manner directly analogous to that used for the ortho-fluoro isomer, using N-acetyl-m-fluoro-DL-phenylalanine, m. p. 154-156°, prepared by Bennett (50). Esterification of this compound (15 g., 0.069 mole) with methanol (85 ml.) and thionyl chloride (8.7 g., 0.072 mole) (103) gave 13 g. (81%) of crude N-acetyl-m-fluoro-DL-phenylalanine methyl ester. After crystallization from isopropyl ether this material had a m. p. of 84-85°.

Enzymatic Resolution of N-acetyl-m-fluoro-DL-phenylalanine Methyl Ester

The equipment and procedure were the same as for the resolution of N-acetyl-o-fluoro-DL-phenylalanine methyl ester. The racemic ester (12.5 g., 0.0541 mole) and α-chymotrypsin (253 mg.) were combined in 800 ml. of a 10% (v/v) methanol-water solution. The hydrolysis ceased after four minutes, 97.2 ml. of 0.258 N aqueous sodium hydroxide having been added, corresponding to approximately 96% hydrolysis of one optical isomer. As with the ortho-fluoro compound the reaction solution was extracted with ethyl acetate.

The organic phase was washed with dilute bicarbonate solution, 1 N aqueous hydrochloric acid, and saturated aqueous sodium chloride. After treatment with anhydrous magnesium sulfate and charcoal, the ethyl acetate was evaporated to dryness under reduced pressure to give 5.8 g. (93%) of the crude D ester.

After four crystallizations from isopropyl ether, N-acetyl-m-fluoro-D-phenylalanine methyl ester had m. p. 83-84°, and $[\alpha]_D^{30} = -(12.1 \pm 0.4)^\circ$ (C, 2.18% in methanol).

N-Acetyl-m-fluoro-L-phenylalanine

The aqueous phase from the resolution of N-acetyl-m-fluoro-DL-phenylalanine methyl ester was concentrated to approximately 20 ml. and acidified to roughly pH 2. The crude N-acetyl-m-fluoro-L-phenylalanine, m. p. 153-156°, was collected in a 5.3 g. (90%) yield. After two crystallizations from water, this material had m. p. 158-160° and $[\alpha]_D^{28} = +(37 \pm 2)^\circ$ (C, 3.91% in ethanol). For the optical antipode of this compound, Bennett and Niemann report m. p. 159-160° and $[\alpha]_D^{25} = -40$ (C, 8% in ethanol) (50).

N-Acetyl-m-fluoro-L-phenylalanine Methyl Ester

Esterification of N-acetyl-m-fluoro-L-phenylalanine and work-up of the product was analogous to that for N-acetyl-o-fluoro-DL-phenylalanine. A crude yield of 4.8 g. (84%) of the ester was obtained from esterification of N-acetyl-m-fluoro-L-phenylalanine (5.2 g., 0.024 mole) with methanol (60 ml.) and thionyl chloride (3.3 g., 0.028 mole). After four crystallizations from isopropyl ether, the product had m. p. 80-81°, $[\alpha]_D^{25} = +(13.0 \pm 0.2)^\circ$, (C, 2.04% in methanol).

Analysis:	calculated	C: 60.2	H: 5.9	N: 5.9
$C_{12}H_{14}NO_3F$ (239)	found	C: 60.5	H: 6.0	N: 6.0

N-Acetyl-p-fluoro-DL-phenylalanine Methyl Ester

Esterification of N-acetyl-p-fluoro-DL-phenylalanine (15 g.) 0.069 mole) with methanol (85 ml.) and thionyl chloride (8.4 g., 0.071 mole) was carried out as described previously. The acylated amino acid, m. p. 150-153°, was a Bennett preparation (50). The crude yield of the desired ester was 12.4 g. (78%). Another preparation, beginning with 4 g. of the acylated amino acid, gave 3 g. (57%) of the desired ester. After one crystallization from isopropyl ether, N-acetyl-p-fluoro-DL-phenylalanine methyl ester had m. p. 64-66°.

Enzymatic Resolution of N-acetyl-p-fluoro-DL-phenylalanine Methyl Ester

The resolution of this compound was performed as described before. A solution of N-acetyl-p-fluoro-DL-phenylalanine methyl ester (12.8 g., 0.0537 mole) in 10% (v/v) methanol-water (800 ml.) was resolved by the action of α-chymotrypsin (156 mg.). The pH of the solution had become almost steady after 17 minutes. A total of 99.5 ml. of 0.258 N aqueous sodium hydroxide had been added, indicating 96% hydrolysis of one optical isomer. As with the two previous resolutions, the ester remaining was extracted with ethyl acetate.

The ethyl acetate extract was washed and dried as described for N-acetyl-o-fluoro-D-phenylalanine methyl ester. Removal of the solvent gave 4.6 g. (71%) of the crude ester. After four crystallizations from isopropyl ether, N-acetyl-p-fluoro-D-phenylalanine

methyl ester had a m. p. of 91-92° and $[\alpha]_D^{25}$ = - (10.3 ± 0.3)° (C, 1.60% in methanol).

N-Acetyl-p-fluoro-L-phenylalanine

Concentration of the aqueous phase from the resolution just described to about 50 ml., followed by acidification to approximately pH 2, gave crude N-acetyl-p-fluoro-L-phenylalanine in a 3.9 g. (65%) yield. After two crystallizations from water, the material gave m. p. 145-146°, $[\alpha]_D^{28}$ = + (35 ± 1)° (C, 3.05% in ethanol). The values reported for the D- antipode of this isomer are: m. p. 142-143°, $[\alpha]_D^{25}$ = - 38.6°, (C, 8% in ethanol) (50).

N-Acetyl-p-fluoro-L-phenylalanine Methyl Ester

As with the other acylated fluoro-phenylalanines, N-acetyl-p-fluoro-L-phenylalanine (3.7 g., 0.016 mole) was esterified with methanol (35 ml.) and thionyl chloride (2.2 g., 0.019 mole). A crude yield of 3.5 g. (85%) N-acetyl-p-fluoro-L-phenylalanine methyl ester was obtained. After four crystallizations from isopropyl ether, the m. p. was 90-91°, and the optical rotation was $[\alpha]_D^{26}$ = + (13.4 ± 0.2)° (C, 2.08% in methanol).

Analysis:	calculated	C: 60.2	H: 5.9	N: 5.9
$C_{12}H_{14}NO_3F$ (239)	found	C: 59.9	H: 5.7	N: 6.1

N-Acetyl-o-chloro-DL-phenylalanine Methyl Ester

The starting material for this synthesis, o-chloro-DL-phenylalanine, had been prepared by Shelberg (110). This amino

acid (10 g., 0.050 mole) was esterified with thionyl chloride (8.0 g., 0.067 mole) and methanol (100 ml.) in the usual way. After removal of the methanol, the amino acid methyl ester hydrochloride was suspended in ethyl acetate (100 ml.) and acylated with acetic anhydride (15 ml., 21 g., 0.40 mole) in the presence of potassium carbonate (55 g., 0.40 mole) in water (100 ml.) (23). The work-up of the acetylated methyl ester was similar to that for N-acetyl-L-phenylalanine methyl ester, described previously. The crude N-acetyl-o-chloro-DL-phenylalanine methyl ester, 6.0 g. (47%), was crystallized from isopropyl ether to give a material having a melting point of 81-83°.

Enzymatic Resolution of N-acetyl-o-chloro-DL-phenylalanine Methyl Ester

The procedure and equipment used for the enzymatic resolution of the acetylated fluoro-DL-phenylalanine methyl esters was used in this case. A solution of N-acetyl-o-chloro-DL-phenylalanine methyl ester (3.38 g., 0.0132 mole) in 400 ml. of 10% (v/v) of methanol-water was resolved by the action of α-chymotrypsin (118 mg.). After 17 minutes, the reaction had slowed considerably. A total of 48.5 ml. of 0.129 M aqueous sodium hydroxide, corresponding to 95% hydrolysis of one optical isomer, had been added. The work-up procedure was analogous to that used for the N-acetyl-o-fluoro-DL-phenylalanine methyl ester resolution.

Work-up of the ethyl acetate extract gave 1.5 g. (84%) crude N-acetyl-o-chloro-D-phenylalanine methyl ester. Four crystallizations from isopropyl ether gave the purified ester, m.p. 100-101° and $[\alpha]_D^{27} = + (18.1 \pm 0.7)^\circ$ (C, 1.94% in methanol).

N-Acetyl-o-chloro-L-phenylalanine

The aqueous phase from the resolution just described was concentrated to a volume of approximately 20 ml. and acidified to about pH 2 with concentrated hydrochloric acid. A yield of 1.2 g. (94%) of N-acetyl-o-chloro-L-phenylalanine was obtained. After one crystallization from water, the material had m.p. 178-180° and $[\alpha]_D^{27}$ = + (15 ± 1)° (C, 1.02% in 0.5 N aqueous sodium hydroxide).

N-Acetyl-o-chloro-L-phenylalanine Methyl Ester

Esterification of N-acetyl-o-chloro-L-phenylalanine (1.0 g., 0.0041 mole) was carried out with thionyl chloride (1.7 g., 0.014 mole) and methanol (50 ml.), as described previously. The crude material was obtained in a yield of 0.83 g. (28%). After four crystallizations from isopropyl ether, it gave a m.p. of 100-101° and $[\alpha]_D^{26}$ = - (18.6 ± 0.5)° (C, 1.0% in methanol).

Analysis:	calculated	C: 56.4	H: 5.5	N: 5.5
$C_{12}H_{14}NO_3Cl$ (256)	found	C: 56.6	H: 5.7	N: 5.7

o-Chloro Benzoic Acid

The oxidation of N-acetyl-o-chloro-L-phenylalanine methyl ester (0.40 g., 0.002 mole) with potassium permanganate (4.0 g., 0.026 mole) was done by a procedure adapted from one given by Vogel (111) for the oxidation of alkyl side chains of aromatic compounds. The resulting crude o-chlorobenzoic acid, obtained

in a 0.042 g. yield (1.3%) was crystallized once from water. The purified acid had a m. p. of 141-141.5°. The mixed melting point with an authentic sample (Eastman Kodak, crystallized once from water) was 140-141°. The melting point recorded in the literature is 140° (112).

N-Acetyl-m-chloro-DL-phenylalanine Methyl Ester

Using the same esterification procedure as before, m-chloro-DL-phenylalanine (9 g., 0.045 mole) was converted to its methyl ester hydrochloride using methanol (100 ml.) and thionyl chloride (7.5 g., 0.063 mole). The parent amino acid had been prepared by Shelberg (110). The methyl ester hydrochloride, suspended in 100 ml. ethyl acetate, was acylated with acetic anhydride (15 ml., 21 g., 0.40 mole) in the presence of potassium carbonate (55 g., 0.40 mole) in water (100 ml.). The work-up was like that for the phenylalanine analog. The crude yield of 4.5 g. (39%) was crystallized once from isopropyl ether to give N-acetyl-m-chloro-DL-phenylalanine methyl ester, m. p. 77-79°.

Enzymatic Resolution of N-acetyl-m-chloro-DL-phenylalanine Methyl Ester

The enzymatic resolution of this compound was like those described previously. The N-acetyl-m-chloro-DL-phenylalanine methyl ester (3.14 g., 0.0123 mole) was resolved using α-chymotrypsin (106 mg.). At the end of 3 minutes, the pH had stopped dropping and 42.8 ml. of 0.129 N aqueous sodium hydroxide

(corresponding to 94% hydrolysis of one isomer) had been added. The resolution solution was extracted with ethyl acetate, as described previously.

Removal of the solvent from the ethyl acetate extract of the resolution solution (after the usual washings, drying with magnesium sulfate and charcoal treatment) gave 1.3 g. (83%) of N-acetyl-m-chloro-D-phenylalanine methyl ester. After four crystallizations from isopropyl ether, this compound gave a m. p. of 88-89° and $[\alpha]_D^{27} = -(16.8 \pm 0.3)^\circ$ (C, 1.51% in methanol).

N-Acetyl-m-chloro-L-phenylalanine

The aqueous phase of the resolution mixture was reduced to a volume of approximately 15 ml. and acidified to about pH 2 with concentrated hydrochloric acid. The product, N-acetyl-m-chloro-L-phenylalanine, was obtained in 1.4 g. (93%) yield. After one crystallization from water it had a m. p. of 161-163° and $[\alpha]_D^{27} = +(38 \pm 1)^\circ$ (C, 1.06% in 0.5 N aqueous sodium hydroxide).

N-Acetyl-m-chloro-L-phenylalanine Methyl Ester

Esterification of N-acetyl-m-chloro-L-phenylalanine (1.3 g., 0.0058 mole) with thionyl chloride (2.0 g., 0.016 mole) and methanol (50 ml.) was effected in the usual way. The crude material, obtained in 1.3 g. (93%) yield, was crystallized four times from isopropyl ether. The purified N-acetyl-m-chloro-L-phenylalanine methyl ester had a m. p. of 86-87° and $[\alpha]_D^{26} = +(15.0 \pm 0.3)^\circ$ (C, 1.36% in methanol).

Analysis:	calculated	C: 56.4	H: 5.5	N: 5.5
$C_{12}H_{14}NO_3Cl$ (256)	found	C: 56.6	H: 5.6	N: 5.6

m-Chlorobenzoic Acid

The procedure followed was like that for the orthochloro analogue. From the oxidation of N-acetyl-m-chloro-L-phenylalanine methyl ester (0.4 g., 0.002 mole) with potassium permanganate (4.0 g., 0.026 mole) was 0.049 g. (15%) m-chlorobenzoic acid, m.p. 154.5-155°, after recrystallization from water. Andrews et al. report m.p. 154° for this compound (112).

N-Acetyl-p-chloro-DL-phenylalanine Methyl Ester

The p-chloro-DL-phenylalanine used in this synthesis was a preparation of Shelberg (110). Esterification of this compound (19.0 g., 0.095 mole) with methanol (200 ml.) and thionyl chloride (14.9 g., 0.125 mole) (103), gave the crude methyl ester hydrochloride. It was suspended in 100 ml. of ethyl acetate and acetylated with acetic anhydride (30 ml., 42 g., 0.80 mole) in the presence of potassium carbonate (110 g., 0.80 mole) in water (200 ml.). A crude yield of 14.5 g., (60%) was obtained. After crystallization from isopropyl ether N-acetyl-p-chloro-DL-phenylalanine methyl ester had a m.p. of 112-114°.

Enzymatic Resolution of N-acetyl-p-chloro-DL-phenylalanine Methyl Ester

The procedure and equipment in this resolution were similar to those for the resolutions described previously. In 15% (v/v) methanol-water (1 liter) were dissolved N-acetyl-p-chloro-DL-phenylalanine methyl ester (11.71 g., 0.0458 mole) and α-chymotrypsin (153 mg.). After 5 hours and 40 minutes, the dropping of pH had slowed considerably, although only 141 ml. of 0.1291 N aqueous sodium hydroxide, corresponding to 79% hydrolysis of one optical isomer, had been added. To avoid excessive hydrolysis of the D antipode by the non-enzymatic reaction, the resolution mixture was extracted with ethyl acetate at this point.

Washing, drying, treatment with charcoal and evaporation to dryness of the ethyl acetate extract gave a crude yield of 6.2 g. (87%, based on the 79% hydrolysis of the L isomer) of the "D" compound. After four crystallizations from isopropyl ether, this material had m. p. 108-109° and $[\alpha]_D^{27} = -(12.4 \pm 0.2)^\circ$ (C, 1.01% in methanol). This rotation is consistent with one calculated assuming that the rotation measured for the L isomer is correct and that the "D" isomer consists of 29% (by weight) of the L isomer and 71% (by weight) of the D isomer. (This is the proportion expected from the amount of base added during the resolution.)

N-Acetyl-p-chloro-L-phenylalanine

The volume of the aqueous phase of the resolution just described was reduced, under partial vacuum and heating, to approximately 20 ml. and acidified to about pH 2 with concentrated

hydrochloric acid. The yield of crude N-acetyl-p-chloro-L-phenylalanine was 2.2 g. (50%, based on 79% hydrolysis of the L isomer in the resolution). After one crystallization from water, this compound had a m. p. of 148-150° and $[\alpha]_D^{27}$ = + (46 ± 3)° (C, 1.08%, in 0.5 N aqueous sodium hydroxide).

N-Acetyl-p-chloro-L-phenylalanine Methyl Ester

Esterification of N-acetyl-p-chloro-L-phenylalanine (2.0 g., 0.0083 mole) was carried out in the usual way, using methanol (50 ml.) and thionyl chloride (1.7 g., 0.014 mole). The crude yield was 1.6 g. (75%) of the ester. After four crystallizations from isopropyl ether, N-acetyl-p-chloro-L-phenylalanine methyl ester had m. p. 117-118° and $[\alpha]_D^{28}$ = + (25.2 ± 0.4)° (C, 1.23% in methanol).

Analysis:	calculated C:	56.4	H:	5.5	N: 5.5
$C_{12}H_{14}NO_3Cl$ (256)	found C:	56.0	H:	5.3	N: 5.2

p-Chlorobenzoic Acid

Oxidation of N-acetyl-p-chloro-L-phenylalanine methyl ester (0.40 g., 0.002 mole) with potassium permanganate (4.0 g., 0.026 mole) was carried out as described before. The yield of p-chlorobenzoic acid was 0.079 g. (25%). After recrystallization from water, it had a m. p. of 242°. A mixture of this acid with an authentic sample (Eastman Kodak, recrystallized from water) melted at 240-241°. The value recorded in the literature is 240° (112).

m-Nitrobenzyl Alcohol

To a solution of m-nitrobenzaldehyde (60 g., 0.40 mole) in methanol (400 ml.) was added a solution of sodium borohydride (8.0 g., 0.21 mole, in 80 ml. 0.5 N aqueous sodium hydroxide). A total of 53 ml. of the sodium borohydride solution was added, while stirring the solution, at a rate such that a reaction temperature of 23-27^{o} was maintained. Further addition of the sodium borohydride solution caused no further reaction, as evidenced by the constancy of the solution temperature. Extraction of the reaction solution with about 600 ml. of diethyl ether, followed by washing of the ethereal extract with 1 N aqueous hydrochloric acid, a saturated aqueous sodium chloride solution, drying with anhydrous magnesium sulfate and evaporation of the ether gave 55 g. (91%) crude m-nitrobenzyl alcohol, a m.p. of 25-26^{o}. Chaiken and Brown give a m.p. of 30^{o} for the pure material (113).

m-Nitrobenzyl Chloride

The m-nitrobenzyl alcohol (55 g., 0.21 mole) was added to a solution of zinc chloride (109 g., 0.80 mole) in concentrated hydrochloric acid (81 g., 0.80 mole) and the mixture was refluxed for 15 minutes. After the mixture was allowed to cool, the solid upper layer was filtered from the solution and extracted with 60-70^{o} petroleum ether. After crystallization from the same solvent, 40 g. (65%) of the product (m.p. 44-46^{o}) were obtained. Norris and Taylor report a m.p. of 45-47^{o} for this compound (114).

Ethyl Acetamido-(m-nitrobenzyl)-malonate

Sodium (2.30 g., 0.10 mole) was dissolved in 160 ml. of ethanol, dried as directed by Vogel (115). After reflux of this solution had begun, dry, twice-crystallized (from methanol) ethyl acetamidomalonate (21.6 g., 0.10 mole) was added, followed by a solution of m-nitrobenzyl chloride (19.5 g., 0.11 mole) in 100 ml. of dry ethanol. After an 18 hour reflux period, the amber solution was cooled in an ice bath, and 21.2 g. (60%) ethyl acetamido-(m-nitrobenzyl)-malonate crystallized from the solution. The compound melted at 157-158°. Gram et al. report a m.p. of 159-160° for this compound (116). Osdene et al. report 159-161° for the same compound (117).

N-Acetyl-m-nitro-DL-phenylalanine

A solution of ethyl acetamido-(m-nitro benzyl)-malonate (20 g., 0.057 mole) in 2.5 N aqueous sodium hydroxide (50 ml.) was refluxed 2.5 hours. The solution was then cooled and 10 ml. concentrated hydrochloric acid was added to it. The acidified solution was refluxed another hour, treated with charcoal and filtered while still hot. Upon cooling and storage in a refrigerator over night, the N-acetyl-m-nitro-DL-phenylalanine crystallized in 13.8 g. (96%) yield. After two crystallizations from water, this material had a m.p. of 147-149°.

Analysis:	calculated	C:	52.4	H:	4.8	N: 11.1
$C_{11}H_{12}N_2O_5$ (252)	found	C:	52.5	H:	4.8	N: 11.1

N-Acetyl-m-nitro-DL-phenylalanine Methyl Ester

Esterification of N-acetyl-m-nitro-DL-phenylalanine (12.5 g., 0.050 mole) with thionyl chloride (6.3 g., 0.053 mole) and methanol (100 ml.) was conducted in the usual way. The work-up, analogous to those described previously, yielded 10 g. (80%) of the crude ester. After crystallization from carbon tetrachloride, N-acetyl-m-nitro-DL-phenylalanine methyl ester had a m. p. of 96-97°.

Enzymatic Resolution of N-Acetyl-m-nitro-DL-phenylalanine Methyl Ester

The procedure and equipment for this resolution were essentially as described for those in the resolution of N-acetyl-o-fluoro-DL-phenylalanine methyl ester. N-Acetyl-m-nitro-DL-phenylalanine methyl ester (6.025 g., 0.0227 mole), in an 18.8% (v/v) methanol-water solution (800 ml.), was resolved by the action of α-chymotrypsin (202 mg.). At the end of 2 minutes, the reaction was complete, 49.8 ml. of 0.232 N aqueous sodium hydroxide having been added. This volume of base corresponds to almost complete hydrolysis (99.7%) of one optical isomer. The work-up was similar to those described previously.

The ethyl acetate extract of the solution from the N-acetyl-m-nitro-DL-phenylalanine methyl ester resolution was washed, dried and evaporated to dryness in the usual way, giving a 2.8 g. (92%) yield of the D ester. After crystallization from carbon tetrachloride (five times), N-acetyl-m-nitro-D-phenylalanine methyl

ester had a m. p. of 99-100° and $[\alpha]_D^{26}$ = - (11.1 ± 0.2)° (C, 2.20% in methanol).

N-Acetyl-m-nitro-L-phenylalanine

The aqueous phase from the resolution just described was concentrated to a volume of approximately 15 ml. and acidified with concentrated hydrochloric acid. The precipitated crude N-acetyl-m-nitro-L-phenylalanine was obtained in a 2.8 g. (98%) yield. After one crystallization from water, this compound had a m. p. of 165-167° and $[\alpha]_D^{28}$ = + (34.9 ± 0.8)° (C, 2.64% in ethanol).

N-Acetyl-m-nitro-L-phenylalanine Methyl Ester

N-Acetyl-m-nitro-L-phenylalanine (2.7 g., 0.011 mole) was esterified with thionyl chloride (1.5 g., 0.012 mole) and methanol (50 ml.) (103). The crude yield of the ester was 2.3 g. (79%). After four crystallizations from carbon tetrachloride, N-acetyl-m-nitro-L-phenylalanine methyl ester had a m. p. of 100-101° and $[\alpha]_D^{26}$ = + (11.0 ± 0.3)° (C, 1.55% in methanol).

Analysis:	calculated	C: 54.1	H: 5.3	N: 10.5
$C_{12}H_{14}N_2O_5$ (266)	found	C: 54.2	H: 5.0	N: 10.6

2-Methyl-4-(o-methoxybenzal)-5-oxazolone

The synthesis of this compound, which was prepared several times, was analogous to one described by Herbst and Shemin (118). The description of a typical synthesis of this compound follows.

o-Anisaldehyde (100 g., 0.73 mole), aceturic acid (80 g., 0.68 mole) (crystallized from water and dried), sodium acetate (38 g., 0.46 mole) (fused and allowed to cool), and acetic anhydride (188 ml., 261 g., 2.56 moles) were mixed thoroughly, brought into solution by heating on the steam bath, and refluxed one hour. The dark red liquid was cooled and placed in the refrigerator over night. The dark brown mass was filtered and washed with cold water, cold ethanol and diethyl ether. The yield was 80 g. (58%) of a bright yellow compound. Yields from similar preparations of this compound ranged from 38 to 61%. The crude products melted over a range of 149-152°. Several attempts to crystallize the compound failed.

α-Acetamido-o-methoxycinnamic Acid

An adaptation of a known oxazolone hydrolysis was employed several times in the preparation of this compound (118). A typical preparation was as follows: 2-methyl-4-(o-methoxybenzal)-5-oxazolone (15 g., 0.069 mole) was dissolved in acetone (150 ml.) and water (50 ml.) added to it. The resulting solution was refluxed four hours, and the acetone distilled off. The resulting mixture of solution and precipitated solid was poured gradually into boiling water and the solid was dissolved. Charcoal was added to the boiling solution which was filtered. Cooling of the solution (total volume of approximately 200 ml.) resulted in crystallization of the product, in a 13 g. (86%) yield. This faintly yellow colored solid was crystallized twice from water to give purified α-acetamido-o-methoxycinnamic acid, white crystals, a m.p. of 214-216°. Yields from similar preparations ranged from 42 to 78%.

Analysis:	calculated	C: 61.3	H: 5.6	N: 6.0
$C_{12}H_{13}NO_4$ (235)	found	C: 61.2	H: 5.7	N: 6.2

N-Acetyl-o-methoxy-DL-phenylalanine

A sodium amalgam reduction similar to the one used here has been described (119). α-Acetamido-o-methoxycinnamic acid (12 g., 0.051 mole) was suspended in water (100 ml.). To this mixture was added, in six portions over a 30 minute period, sodium amalgam made by dissolving sodium (6.0 g., 0.26 mole) in mercury (175 g.). The biphasic mixture was stirred 4 hours. At the end of that period, the solution did not decolorize a potassium permanganate solution. The aqueous phase was decanted from the mercury phase, treated with charcoal, filtered and acidified. N-Acetyl-o-methoxy-DL-phenylalanine was obtained in a 10 g. (86%) yield. Similar reductions of this compound gave 66-96% yields. After two crystallizations from water, this compound melted at 166-167°.

Analysis:	calculated	C: 60.8	H: 6.4	N: 5.9
$C_{12}H_{15}NO_4$ (237)	found	C: 61.0	H: 6.4	N: 5.8

N-Acetyl-o-methoxy-DL-phenylalanine Methyl Ester

The synthesis of this compound was like the esterifications described previously. Esterification of N-acetyl-o-methoxy-DL-phenylalanine (56 g., 0.24 mole) with thionyl chloride (32 g., 0.26 mole) and methanol (600 ml.) gave 48 g. (80%) of the ester. After crystallization from isopropyl ether, this compound melted at 88-89°.

Enzymatic Resolution of N-acetyl-o-methoxy-DL-phenylalanine Methyl Ester

The procedure used in this case was similar to those described previously. N-Acetyl-o-methoxy-DL-phenylalanine methyl ester (15.2 g., 0.0606 mole) was dissolved in 15% (v/v) methanol-water (1 liter) and α-chymotrypsin (368 mg.) added to it. At the end of 25 minutes, 129 ml. of 0.231 N aqueous sodium hydroxide, corresponding to almost complete (99%) hydrolysis of one optical antipode, had been added. The work-up was similar to those described previously. This resolution is typical of several which were used to obtain the optical isomers of this compound. All of them were carried out to a point corresponding to at least 93% hydrolysis of one optical isomer.

The ethyl acetate extract from the resolution just described was washed, dried, and evaporated to dryness as described before. A crude yield of 7.3 g. (96%) of N-acetyl-o-methoxy-D-phenylalanine methyl ester was obtained. After four crystallizations from isopropyl ether, this material had a m. p. of 79-80° and $[\alpha]_D^{26}$ = + (4.3 ± 0.2)° (C, 2.40% in methanol).

N-Acetyl-o-methoxy-L-phenylalanine

The aqueous phase of the resolution just described was reduced to a volume of approximately 25 ml. and acidified with concentrated hydrochloric acid. The crude N-acetyl-o-methoxy-L-phenylalanine was collected in 6.1 g. (84%) yield. Similar yields were obtained in the other resolutions of the parent DL ester. After

crystallization from water, this compound had a m. p. of 173-174° and $[\alpha]_D^{25}$ = + (16 ± 1)° (C, 1.29% in 0.5 N aqueous sodium hydroxide).

N-Acetyl-o-methoxy-L-phenylalanine Methyl Ester

Esterification with thionyl chloride (3.6 g., 0.030 mole) and methanol (50 ml.) of N-acetyl-o-methoxy-L-phenylalanine (6.0 g., 0.025 mole) was accomplished by the Brenner-Huber method (103). A crude yield of 5.0 g. (78%) was obtained. After four crystallizations from isopropyl ether, the product, N-acetyl-o-methoxy-L-phenylalanine methyl ester melted at 77-78° and had $[\alpha]_D^{25}$ = - (4.7 ± 0.2)° (C, 2.10% in methanol).

Analysis:	calculated	C: 62.1	H: 6.8	N: 5.6
$C_{13}H_{17}NO_4$ (251)	found	C: 62.1	H: 6.8	N: 5.7

o-Hydroxy-L-phenylalanine

The methyl ether of N-acetyl-o-methoxy-L-phenylalanine was cleaved by the action of boiling hydriodic acid, with simultaneous hydrolysis of the α-acetamido group. N-Acetyl-o-methoxy-L-phenylalanine (20 g., 0.084 mole) was added to hydriodic acid (200 ml. of 57% hydriodic acid, 1.52 moles). The mixture was refluxed 5 hours. At the end of that period, the red colored solution was evaporated to dryness under reduced pressure and the residue dissolved in 75 ml. of water. This solution was extracted repeatedly with diethyl ether. The aqueous phase was treated with charcoal and filtered. The acid solution was neutralized with concentrated aqueous ammonium

hydroxide to pH 8 and allowed to crystallize. A total of 9.0 g. (59%) of o-hydroxy-L-phenylalanine was collected.

N-Acetyl-o-hydroxy-L-phenylalanine Methyl Ester

Brenner-Huber (103) esterification with thionyl chloride (6.6 g., 0.056 mole), methanol (80 ml.) and o-hydroxy-L-phenylalanine (8.9 g., 0.049 mole), produced the corresponding amino acid methyl ester hydrochloride. This compound was added to a mixture of sodium acetate (7.6 g., 0.093 mole) and acetic anhydride (41 ml., 57 g., 0.56 mole), and the usual acetylation procedure followed. A yield of 9.1 g. (78%) of N-acetyl-o-hydroxy-L-phenylalanine methyl ester was produced. After four crystallizations from diethyl ether, this compound melted at 82-83° and had $[\alpha]_D^{26} = -(7.7 \pm 0.2)^\circ$ (C, 1.82% in methanol).

Analysis:	calculated	C: 60.8	H: 6.4	N: 5.9
$C_{12}H_{15}NO_4$ (237)	found	C: 60.5	H: 6.0	N: 5.9

N-Acetyl-o-ethoxy-L-phenylalanine

The procedure for the preparation of alkyl ethers of N-acetyl-L-tyrosine was adapted for synthesis of this compound (29). To the yellow colored solution of N-acetyl-o-hydroxy-L-phenylalanine methyl ester (2.4 g., 0.010 mole) in 4 N aqueous potassium hydroxide (12 ml.) was added redistilled ethyl iodide (4.0 ml., 6.1 g., 0.039 mole) and ethanol (10 ml.). The biphasic mixture was emulsified by stirring and kept in that condition at 45-50° for 12 hours. After cooling the mixture, it was extracted with 350 ml. diethyl ether.

The ethereal extract was concentrated to a volume of 30 ml., to which 10 ml. of water was added. After the rest of the diethyl ether was removed, the two aqueous solutions were combined, treated with charcoal, filtered and acidified. The yield of N-acetyl-o-ethoxy-L-phenylalanine was 1.4 g. (56%). After crystallization from water, this material had a m. p. of 131-133° and $[\alpha]_D^{26} = + (7.0 \pm 0.5)^\circ$ (C, 2.17% in 0.5 N aqueous sodium hydroxide).

N-Acetyl-o-ethoxy-L-phenylalanine Methyl Ester

Esterification with thionyl chloride (0.66 g., 0.0056 mole) and methanol (25 ml.) was performed in the usual way on N-acetyl-o-ethoxy-L-phenylalanine (1.2 g., 0.005 mole). The corresponding ester was obtained in 1.0 g. (71%) yield. After four crystallizations from isopropyl ether, N-acetyl-o-ethoxy-L-phenylalanine methyl ester melted at 86-87° and had $[\alpha]_D^{26} = - (12.6 \pm 0.4)^\circ$ (C, 1.07% in methanol).

Analysis:	calculated	C: 63.4	H: 7.2	N: 5.3
$C_{14}H_{19}NO_4$ (265)	found	C: 63.3	H: 7.0	N: 5.3

o-Hydroxy-D-phenylalanine

In a manner analogous to that used for the preparation of the L isomer, N-acetyl-o-methoxy-D-phenylalanine methyl ester (10 g., 0.040 mole) was added to a hydriodic acid solution (100 ml. of 57% hydriodic acid, 0.76 mole) and the mixture was refluxed for 5 hours. The work-up was the same as for the L antipode. A total yield of 3.9 g. (54%) of o-hydroxy-D-phenylalanine was obtained. After

crystallization from water this material had $[\alpha]_D^{27} = + (5.7 \pm 0.4)^o$ (C, 2.81% in 1 N HCl) and $[\alpha]_D^{27} = + (23 \pm 1)^o$ (C, 0.56% in water). Since the rotation is lower in acid than in water, the D configuration is established (49).

N-Acetyl-o-hydroxy-D-phenylalanine Methyl Ester

Esterification of o-hydroxy-D-phenylalanine (8.0 g., 0.044 mole) by the usual Brenner-Huber (103) method with thionyl chloride (5.3 g., 0.047 mole) and methanol (60 ml.) gave the methyl ester hydrochloride. This was added to a mixture of sodium acetate (6.9 g., 0.084 mole) and acetic anhydride (40 ml., 56 g., 0.54 mole) to acetylate it. The crude N-acetyl-o-hydroxy-L-phenylalanine methyl ester was obtained in 7.3 g. (70%) yield. After four crystallizations from diethyl ether, this compound melted at 84-85° and had $[\alpha]_D^{26} = + (7.8 \pm 0.2)^o$ (C, 1.79% in methanol).

m-Methoxybenzaldehyde

The procedure of Icke et al. was used for the preparation of this compound (120). A solution of m-hydroxybenzaldehyde (61 g., 0.50 mole) in 2 N aqueous sodium hydroxide (350 ml.) was methylated with dimethyl sulfate (60 ml., 83 g., 0.66 mole), which was added, dropwise and with stirring, at such a rate that the temperature of the reaction solution was maintained at 40-45°. After all the dimethyl sulfate was added, the reaction mixture was stirred five minutes, then more methyl sulfate (30 ml., 42 g., 0.33 mole) and 2 N aqueous sodium hydroxide (150 ml.) were added. After a 45 minute stirring period at 45°, the solution was cooled and extracted with diethyl ether

(2 lb.). The ethereal extract was dried, treated with charcoal, filtered, and evaporated to dryness. The residue was distilled at 2 mm. Hg pressure. The boiling point at this pressure was 75-78° (uncorrected). Icke et al. report a boiling point of 88-90° at 3 mm. Hg (120). The yield was 58 g. (85%). Other preparations gave 76-91% yields.

2-Methyl-4-(m-methoxybenzol)-5-oxazolone

Several preparations of this compound were made, using an adaptation of the method of Herbst and Shemin (118). In a typical preparation, m-methoxybenzaldehyde (89 g., 0.65 mole), aceturic acid (72 g., 0.61 g.) (crystallized from water, dried), sodium acetate (35 g., 0.43 mole) (fused and cooled), and acetic anhydride (170 ml., 236 g., 2.31 mole) were mixed and heated on the steam bath to form a solution. The resulting solution was refluxed one hour, cooled overnight in the refrigerator, and filtered from the yellow crystals which appeared. The crystals were washed with cold water, cold ethanol and diethyl ether, then dried, giving 61 g. (43%) of the oxazolone, m. p. 109-111°. Yields of 57-77% were obtained in other preparations. Several attempts to crystallize this compound failed.

α-Acetamido-m-methoxycinnamic Acid

Hydrolysis of the crude oxazolone just described was performed by dissolving 2-methyl-4-(m-methoxybenzal)-5-oxazolone (13 g., 0.060 mole) in acetone (110 ml.), adding water (45 ml.) and

refluxing the solution for four hours. The acetone was removed by distillation, more water (100 ml.) added and the mixture boiled to dissolve the product. The hot solution was treated with charcoal, filtered and allowed to crystallize. A yield of 10 g. (75%) of α-acetamido-m-methoxycinnamic acid was obtained. This procedure was applied several times, giving yields of 73-90% and was an adaptation of the Herbst-Shemin method (118). After crystallization from water, the product melted at 171-172°.

Analysis:	calculated	C: 61.3	H: 5.6	N: 6.0
$C_{12}H_{13}NO_4$ (335)	found	C: 61.5	H: 5.7	N: 6.3

N-Acetyl-m-methoxy-DL-phenylalanine

α-Acetamido-m-methoxycinnamic acid (10 g., 0.042 mole), suspended in 100 ml. of water was reduced by an amalgam consisting of sodium (5.0 g., 0.22 mole) dissolved in mercury (175 g.). The amalgam was added in 3 portions over a 20 minute time interval. The mixture was stirred five hours, and the aqueous layer separated. After treatment with charcoal and filtration, the aqueous phase was acidified with concentrated hydrochloric acid. A yield of 7.5 g. (75%) N-acetyl-m-methoxy-DL-phenylalanine was obtained. Yields of 70-90% were obtained in other applications of this method, which is one adapted from a sodium amalgam reduction described in the literature (119). The compound, after crystallization from water, melted at 137-139°.

Analysis:	calculated	C: 60.8	H: 6.4	N: 5.9
$C_{12}H_{15}NO_4$ (237)	found	C: 60.3	H: 6.2	N: 6.1

N-Acetyl-m-methoxy-DL-phenylalanine Methyl Ester

N-Acetyl-m-methoxy-DL-phenylalanine (7.0 g., 0.030 mole) was esterified with thionyl chloride (5.0 g., 0.042 mole) and methanol (25 ml.) in the usual way. A yield of 5.0 g. (70%) of N-acetyl-m-methoxy-DL-phenylalanine methyl ester was obtained. The compound melted at 72-73°, after crystallization from isopropyl ether. Yields of 72-83% were obtained in other preparations.

Enzymatic Resolution of N-acetyl-m-methoxy-DL-phenylalanine Methyl Ester

N-Acetyl-m-methoxy-DL-phenylalanine methyl ester (8.03 g., 0.0320 mole) was dissolved in a 15% (v/v) methanol-water solution (500 ml.) and was resolved by the action of α-chymotrypsin (58.8 mg.). The hydrolysis reaction had almost stopped after 35 minutes. The amount of base added to maintain the pH between 7.5 and 8.0 was 74.0 ml. of 0.213 N aqueous sodium hydroxide, 99% of the amount corresponding to complete hydrolysis of one optical isomer. The work-up of the products was similar to that for previous resolutions, as were the equipment and the rest of the procedure.

The usual washing, drying, treatment with charcoal, and evaporation to dryness of the ethyl acetate extract from the resolution gave 3.5 g. (88%) of N-acetyl-m-methoxy-D-phenylalanine methyl ester. This compound melted at 95-96° and had $[\alpha]_D^{26}$ = - (17.1 ± 0.2)° (C, 2.10% in methanol), after four crystallizations from isopropyl ether.

N-Acetyl-m-methoxy-L-phenylalanine

Concentration of the aqueous phase from the above resolution to a volume of about 70 ml., followed by acidification to a pH of approximately 2 with concentrated hydrochloric acid, gave 3.1 g. (81%) of the product. After crystallization from water, N-acetyl-m-methoxy-L-phenylalanine had a m. p. of 148-150° and $[\alpha]_D^{28}$ = + (43.0 ± 0.7)° (C, 3.16% in ethanol).

N-Acetyl-m-methoxy-L-phenylalanine Methyl Ester

The methyl ester of N-acetyl-m-methoxy-L-phenylalanine was prepared by the usual Brenner-Huber (103) method. The acetylated amino acid (2.1 g., 0.0089 mole) was esterified with thionyl chloride (1.7 g., 0.014 mole) and methanol (20 ml.). The yield of N-acetyl-m-methoxy-L-phenylalanine methyl ester was 1.6 g. (73%). After four crystallizations from isopropyl ether, this compound melted at 96-97° and had $[\alpha]_D^{27}$ = + (17.2 ± 0.5)° (C, 2.56% in methanol).

Analysis:	calculated	C: 62.2	H: 6.8	N: 5.6
$C_{13}H_{17}NO_4$ (251)	found	C: 62.1	H: 6.8	N: 5.6

m-Hydroxy-L-phenylalanine

Simultaneous cleavage of the methyl ether and the acetamido group of N-acetyl-m-methoxy-L-phenylalanine (10 g., 0.040 mole) with refluxing hydriodic acid (100 ml. of 57% hydriodic acid, 0.76 mole) gave m-hydroxy-L-phenylalanine in 5.6 g. (78%) yield. In other similar preparations, 74-81% yields were obtained.

N-Acetyl-m-hydroxy-L-phenylalanine Methyl Ester

Esterification of m-hydroxy-L-phenylalanine (5.6 g., 0.031 mole) with thionyl chloride (4.2 g., 0.035 mole) and methanol (50 ml.) gave the amino acid methyl ester hydrochloride. That compound was acetylated with a mixture of acetic anhydride (26 ml., 36 g., 0.30 mole) and sodium acetate (4.7 g., 0.057 mole), as described before. A yield of 4.9 g. (67%) of N-acetyl-m-hydroxy-L-phenylalanine methyl ester was obtained. In other preparations, the yields ranged from 56-74%. This acetylated methyl ester was purified by four crystallizations from diethyl ether, giving white crystals melting at 94-95° and with $[\alpha]_D^{25} = + (16.0 \pm 0.2)^\circ$ (C, 1.81% in methanol).

Analysis:	calculated	C: 60.7	H: 6.4	N: 5.9
$C_{12}H_{15}NO_4$ (237)	found	C: 60.5	H: 6.1	N: 5.9

N-Acetyl-m-ethoxy-L-phenylalanine

The procedure for making the ethyl ether of N-acetyl-m-hydroxy-L-phenylalanine and work-up of the product were similar to that for the ortho analogue (29). The yellow solution made by dissolving N-acetyl-m-hydroxy-L-phenylalanine methyl ester (3.6 g., 0.024 mole) in 4 N aqueous potassium hydroxide (18 ml.) was treated with ethanol (15 ml.) and redistilled ethyl iodide (6.0 ml., 9.1 g., 0.059 mole) to give N-acetyl-m-ethoxy-L-phenylalanine in 2.9 g. (76%) yield. After crystallization from water, this compound melted at 144-145° and had $[\alpha]_D^{25} = + (40.2 \pm 0.3)^\circ$ (C, 3.21% in ethanol).

N-Acetyl-m-ethoxy-L-phenylalanine Methyl Ester

The methyl ester of the previous compound was prepared from N-acetyl-m-ethoxy-L-phenylalanine (2.5 g., 0.010 mole), thionyl chloride (1.3 g., 0.011 mole) and methanol (50 ml.) by the usual method. A yield of 2.0 g. (74%) of the ester was obtained. After four crystallizations from isopropyl ether, N-acetyl-m-ethoxy-L-phenylalanine methyl ester melted at 60-61° and had $[\alpha]_D^{26} = + (15.0 \pm 0.5)^\circ$ (C, 1.06% in methanol).

Analysis:	calculated C: 63.4	H: 7.2	N: 5.3
$C_{14}H_{19}NO_4$ (265)	found C: 63.3	H: 7.0	N: 5.3

N-Acetyl-m-isopropyloxy-L-phenylalanine

To a solution of N-acetyl-m-hydroxy-L-phenylalanine methyl ester (3.6 g., 0.024 mole) in 4 N aqueous potassium hydroxide (18 ml.) were added isopropyl alcohol (18 ml.) and redistilled isopropyl iodide (6.0 ml., 9.0 g., 0.053 mole). The reaction conditions and work-up were similar to those in the N-acetyl-o-ethoxy-L-phenylalanine synthesis. A yield of 1.3 g. (32%) N-acetyl-m-isopropyloxy-L-phenylalanine was obtained. After crystallization from water this material melted at 111-113°.

N-Acetyl-m-isopropyloxy-L-phenylalanine Methyl Ester

Esterification of N-acetyl-m-isopropyloxy-L-phenylalanine (1.2 g., 0.0045 mole) with thionyl chloride (0.66 g., 0.0055 mole) and methanol (25 ml.), gave the methyl ester in 0.9 g. (70%) yield.

After four crystallizations from isopropyl ether, this ester melted at 77-78° and had $[\alpha]_D^{27}$ = + (19.2 ± 0.6)° (C, 1.68% in methanol).

Analysis:	calculated	C: 64.5	H: 7.6	N: 5.0
$C_{15}H_{21}NO_4$ (279)	found	C: 64.5	H: 7.6	N: 5.1

m-Hydroxy-D-phenylalanine

Refluxing N-acetyl-m-methoxy-D-phenylalanine methyl ester (10 g., 0.042 mole) with hydriodic acid (100 ml. of 57% hydriodic acid, 0.76 mole) gave 4.5 g. (59%) of m-hydroxy-D-phenylalanine. Reaction conditions and work-up were the same as with the ortho isomer. Crystallization from water gave the purified compound with $[\alpha]_D^{27}$ = + (28 ± 2)° (C, 1.08% in water). Sealock et al. report $[\alpha]_D^{25}$ = + 30° (C, 0.90% in water) for this compound (48). It was also found that $[\alpha]_D^{27}$ = + (17 ± 2)° (C, 1.08% in N aqueous hydrochloric acid). The lower rotation in acid solution also establishes the D configuration (120).

N-Acetyl-m-hydroxy-D-phenylalanine Methyl Ester

Thionyl chloride (1.7 g., 0.014 mole) and methanol (20 ml.) were used to esterify m-hydroxy-D-phenylalanine (1.5 g., 0.0083 mole). The resulting methyl ester hydrochloride was acetylated with acetic anhydride (8.4 ml., 12 g., 0.12 mole) and sodium acetate (1.3 g., 0.016 mole) to give 1.4 g. (70%) of product. Four crystallizations from diethyl ether gave purified N-acetyl-m-hydroxy-D-phenylalanine methyl ester, melting at 96-97° and with $[\alpha]_D^{22}$ = - (13.5 ± 0.2)° (C, 1.82% in methanol).

α-Acetamido-3, 5-dimethoxycinnamic Acid

The Herbst-Shemin (118) method was applied to the preparation of 2-methyl-4-(3, 5-dimethoxybenzal)-5-oxazolone. A mixture of 3, 5-dimethoxybenzaldehyde (10 g., 0.060 mole), crystallized aceturic acid (7.0 g., 0.060 mole), acetic anhydride (15 ml., 21 g., 0.20 mole), and sodium acetate (3.7 g., 0.045 mole) gave a black colored product which resisted purification. All this product was dissolved in acetone (100 ml.), water (30 ml.) was added and the solution was refluxed four hours. Upon removal of the acetone by distillation, addition of more water (60 ml.), boiling of the residue and water to solution and allowing crystallization of the product to occur, 5 g. (36%) of α-acetamido-3, 5-dimethoxycinnamic acid, melting at 180-182°, was obtained.

N-Acetyl-3, 5-dimethoxy-DL-phenylalanine

The entire yield of the previous synthesis of α-acetamido-3, 5-dimethoxycinnamic acid (5.0 g., 0.019 mole) was suspended in water (45 ml.) and reduced with an amalgam consisting of sodium (2.7 g., 0.12 mole) dissolved in mercury (90 g.). N-Acetyl-3, 5-dimethoxy-DL-phenylalanine, m. p. 158-160°, was obtained in a 4.0 g. (80%) yield.

Analysis:	calculated	C: 58.4	H: 6.4	N: 5.2
$C_{13}H_{17}NO_5$ (267)	found	C: 58.6	II: 6.5	N: 5.1

N-Acetyl-3, 5-dimethoxy-DL-phenylalanine Methyl Ester

The N-acetyl-3, 5-dimethoxy-DL-phenylalanine (3.9 g., 0.015 mole) was esterified with thionyl chloride (2.0 g., 0.017 mole) and methanol (40 ml.). A yield of 2.9 g. (69%) of the ester was obtained. This compound melted at 111-112° after crystallization from isopropyl ether.

Enzymatic Resolution of N-acetyl-3, 5-dimethoxy-DL-phenylalanine Methyl Ester

The resolution procedure described before was employed in this case. To a 15% (v/v) methanol-water (1 liter) solution of N-acetyl-3, 5-dimethoxy-DL-phenylalanine methyl ester (2.89 g., 0.0103 mole) was added α-chymotrypsin (102 mg.). The pH of this solution was nearly constant with time after 28 minutes, 35.0 ml. of 0.141 N aqueous sodium hydroxide having been added. The work-up of the two products was like those for the previous resolutions.

Evaporation to dryness of the washed, dried, and charcoal treated ethyl acetate extract of the resolution just described gave 1.2 g. (80%) of N-acetyl-3, 5-dimethoxy-D-phenylalanine methyl ester. This compound, after four crystallizations from isopropyl ether, melted at 114-115° and had $[\alpha]_D^{24} = -(14.5 \pm 0.4)^\circ$ (C, 1.10% in methanol).

N-Acetyl-3, 5-dimethoxy-L-phenylalanine

The volume of the aqueous phase of the resolution just described was reduced under partial vacuum to about 25 ml. and

acidified with concentrated hydrochloric acid to a low pH. A yield of 1.1 g. (79%) of N-acetyl-3,5-dimethoxy-L-phenylalanine was obtained. After crystallization from water, this compound melted at 143-144° and had $[\alpha]_D^{27}$ = + (42.0 ± 0.2)° (C, 4.07% in 0.5 N aqueous sodium hydroxide).

N-Acetyl-3,5-dimethoxy-L-phenylalanine Methyl Ester

Esterification of N-acetyl-3,5-dimethoxy-L-phenylalanine (1.0 g., 0.0038 mole) with thionyl chloride (0.66 g., 0.056 mole) and methanol (25 ml.) gave 0.80 g. (79%) of the ester. This ester melted at 115-116° and had $[\alpha]_D^{27}$ = + (18.0 ± 0.5)° (C, 1.09% in methanol), after four crystallizations from isopropyl ether.

Analysis:	calculated	C: 59.8	H: 6.8	N: 5.0
$C_{14}H_{19}NO_5$ (281)	found	C: 59.9	H: 6.8	N: 4.9

2-Methyl-4-(p-methylbenzal)-5-oxazolone

A mixture of p-methylbenzaldehyde (100 g., 0.83 mole), crystallized aceturic acid (91 g., 0.78 mole), freshly fused and cooled sodium acetate (43 g., 0.52 mole) and acetic anhydride (215 ml., 299 g., 2.93 moles) were heated to effect solution, then refluxed one hour. After washing with cold water, ethanol and diethyl ether, a yield of 78 g. (50%) of the dried oxazolone (m. p. 131-136°) was obtained. Okuda and Fujii report a m. p. of 133-135° (121).

α-Acetamido-p-methylcinnamic Acid

A four hour reflux of a solution of the oxazolone (78 g., 0.39 mole) in acetone (820 ml.) and water (320 ml.) resulted in a 50 g. (58%) yield of the cinnamic acid derivative, after the usual removal of acetone and addition of more water (200 ml.). The purified (by water crystallization) α-acetamido-p-methyl cinnamic acid melted at 227-229°.

Analysis:	calculated	C: 65.7	H: 6.0	N: 6.4
$C_{12}H_{13}NO_3$ (219)	found	C: 65.8	H: 5.9	N: 6.0

N-Acetyl-p-methyl-DL-phenylalanine

Reduction of α-acetamido-p-methylcinnamic acid (45 g., 0.20 mole) suspended in water (490 ml.) with an amalgam made by dissolving sodium (30 g., 1.30 mole) in mercury (990 g.) gave 40 g. (90%) of N-acetyl-p-methyl-DL-phenylalanine (23). After crystallization from water, this compound melted at 170-172°. A melting point of 164° has been reported for this compound (121).

Analysis:	calculated	C: 65.1	H: 6.8	N: 6.3
$C_{12}H_{15}NO_3$ (221)	found	C: 65.1	H: 6.9	N: 5.9

N-Acetyl-p-methyl-DL-phenylalanine Methyl Ester

The esterification of N-acetyl-p-methyl-DL-phenylalanine (39 g., 0.18 mole) with methanol (400 ml.) and thionyl chloride (25 g., 0.21 mole) gave 33 g. (78%) of the ester. After crystallization from isopropyl ether, it melted at 128-130°.

Enzymatic Resolution of N-acetyl-p-methyl-DL-phenylalanine Methyl Ester

The equipment and general method used in previously described resolutions were used in this case. N-acetyl-p-methyl-DL-phenylalanine methyl ester (5.01 g., 0.0213 mole) was dissolved in 17.5% (v/v) methanol-water, α-chymotrypsin (209 mg.) added to the solution and the pH maintained between 7.5 and 8.0 with 0.226 N aqueous sodium hydroxide. The pH became nearly constant after one hour and 40 minutes, 42.4 ml. of the base solution (corresponding to 90% hydrolysis of one isomer) having been added. The work-up was similar to those described previously.

Evaporation to dryness of the washed, dried, and charcoal treated ethyl acetate extract of the resolution mixture gave 2.3 g. (92%) of N-acetyl-p-methyl-D-phenylalanine methyl ester. This compound, following purification by four crystallizations from isopropyl ether, melted at 93-94° and had $[\alpha]_D^{27} = -(20.7 \pm 0.3)^\circ$ (C, 1.40% in methanol).

N-Acetyl-p-methyl-L-phenylalanine

Concentration under reduced pressure of the aqueous phase of the resolution just described to a volume of about 25 ml., followed by adjustment of the solution pH to about 3, gave 3.2 g. (78%) N-acetyl-p-methyl-L-phenylalanine. Crystallization from water gave the purified material, m. p. 171-173° and $[\alpha]_D^{27} = +(47 \pm 4)^\circ$ (C, 3.52% in 0.5 N aqueous sodium hydroxide).

N-Acetyl-p-methyl-L-phenylalanine Methyl Ester

Esterification of N-acetyl-p-methyl-L-phenylalanine methyl ester (3.1 g., 0.014 mole) with thionyl chloride (3.0 g., 0.025 mole) and methanol (35 ml.) gave 2.1 g. (68%) of N-acetyl-p-methyl-L-phenylalanine methyl ester. After four crystallizations from isopropyl ether, this compound melted at 93-94° and had $[\alpha]_D^{26}$ = + (23.3 ± 0.3)° (C, 1.80% in methanol).

Analysis:	calculated	C: 66.5	H: 7.2	N: 6.0
$C_{13}H_{17}NO_3$ (235)	found	C: 66.4	H: 7.0	N: 6.0

α-Acetamido-m-methylcinnamic Acid

A solution made by heating a mixture of m-methyl-benzaldehyde (19 g., 0.16 mole), crystallized aceturic acid (19 g., 0.16 mole), fused and cooled sodium acetate (8.6 g., 0.10 mole), and acetic anhydride (43 ml., 60 g., 0.60 mole) was refluxed one hour. The initially pale yellow solution turned black and no product could be isolated from it. The black residue was refluxed four hours in a solution of acetone (200 ml.) and water (80 ml.). The product which was obtained by removing the acetone and adding more water (40 ml.) was isolated in a 2.5 g. (7%) yield. This product, α-acetamido-m-methylcinnamic acid, melted at 210-218°.

N-Acetyl-m-methyl-DL-phenylalanine

An amalgam made by dissolving sodium (1.6 g., 0.070 mole) in mercury (52 g.) was added to a suspension of α-acetamido-m-methylcinnamic acid (2.3 g., 0.010 mole) in water (25 ml.) and the

mixture was stirred four hours. A yield of 1.7 g. (74%) N-acetyl-m-methyl-DL-phenylalanine was obtained. After crystallization from water, this compound melted at 150-151°.

Analysis:	calculated	C: 65.1	H: 6.8	N: 6.3
$C_{12}H_{15}NO_3$ (221)	found	C: 65.2	H: 6.9	N: 5.9

N-Acetyl-m-methyl-DL-phenylalanine Methyl Ester

Esterification of N-acetyl-m-methyl-DL-phenylalanine (1.6 g., 0.0072 mole) with thionyl chloride (1.3 g., 0.011 mole) and methanol (25 ml.) gave 1.1 g. (63%) N-acetyl-m-methyl-DL-phenylalanine methyl ester. After crystallization from isopropyl ether, this compound melted at 51-53°.

Enzymatic Resolution of N-acetyl-m-methyl-DL-phenylalanine Methyl Ester

This resolution was conducted in a manner similar to previous resolutions. A solution of N-acetyl-m-methyl-DL-phenylalanine methyl ester (1.02 g., 0.00434 mole) in 8.8% (v/v) methanol-water (250 ml.) was treated with α-chymotrypsin (153 mg.). After 2.5 minutes, 15.4 ml. of 0.141 M aqueous sodium hydroxide (corresponding to 98% hydrolysis of one optical isomer) had been added and the pH of the solution remained constant after this time. The work-up procedure has been described previously.

Evaporation of the ethyl acetate extract from the resolution to dryness after washing, drying and charcoal treatment gave 0.48 g. (94%) N-acetyl-m-methyl-D-phenylalanine methyl ester. After

purification by four crystallizations from isopropyl ether, this compound melted at 67-68° and had $[\alpha]_D^{27} = -(19.4 \pm 0.5)^\circ$ (C, 1.03% in methanol).

N-Acetyl-m-methyl-L-phenylalanine

The aqueous phase from the resolution was concentrated to a volume of approximately 25 ml. and made strongly acid with concentrated hydrochloric acid. A yield of 0.49 g. (96%) of N-acetyl-m-methyl-L-phenylalanine was obtained. After crystallization from water, this compound melted at 159-160° and had $[\alpha]_D^{28} = +(39 \pm 2)^\circ$ (C, 4.92% in 0.5 N aqueous sodium hydroxide).

N-Acetyl-m-methyl-L-phenylalanine Methyl Ester

Esterification of N-acetyl-m-methyl-L-phenylalanine (0.45 g., 0.0020 mole) with thionyl chloride (0.66 g., 0.0056 mole) and methanol (20 ml.) gave the methyl ester in 0.43 g. (91%) yield. After four crystallizations from isopropyl ether, N-acetyl-m-methyl-L-phenylalanine methyl ester melted at 66-67° and had $[\alpha]_D^{26} = +(20.7 \pm 0.4)^\circ$ (C, 1.14% in methanol).

Analysis:	calculated	C: 66.3	H: 7.3	N: 5.9
$C_{13}H_{17}NO_3$ (235)	found	C: 66.3	H: 7.4	N: 5.8

N-Acetyl-L-leucine Methyl Ester

The preparation of this compound has been described in detail in the literature (34, 108). The literature preparation was

used to obtain this methyl ester in a yield of 73%. After several crystallizations from isopropyl ether, this compound melted at 44-46° and had $[\alpha]_D^{25} = -(56.5 \pm 0.2)^\circ$ (C, 3.16% in water). Hein, et al. report a m. p. of 46-47° and $[\alpha]_D^{25} = -(56.8 \pm 1.0)^\circ$ (C, 3% in water) (34).

p-Methoxyhippuric Acid

The method of Acheson et al. was used for the synthesis of this compound (122). To a solution of glycine (19 g., 0.25 mole) in 0.5 N aqueous sodium hydroxide (80 ml.) was added, over a 10 minute period, p-methoxybenzoyl chloride (9.8 g., 0.058 mole) dissolved in diethyl ether (24 ml.). The reaction mixture was allowed to stir 50 minutes. As the reaction progressed, enough N aqueous sodium hydroxide (about 50 ml.) was added to maintain a pH of 8. The solution was acidified and 3.5 g. (27%) p-methoxyhippuric acid was collected. This material, after crystallization from water, melted at 169-170°. The value reported by Quick is 170° (123).

Methyl p-methoxyhippurate

Esterification of p-methoxyhippuric acid (3.2 g., 0.015 mole) with thionyl chloride (20 g., 0.017 mole) and methanol (50 ml.) gave 2.9 g. (89%) of the ester. After crystallization from isopropyl ether (four times) this compound melted at 101-102°.

Analysis:	calculated	C: 59.2	H: 5.9	N: 6.3
$C_{11}H_{13}NO_4$ (223)	found	C: 59.8	H: 6.1	N: 6.3

m-Methoxybenzoic Acid

Sodium (6.0 g., 0.26 mole) was added to ethanol (90 ml.), followed by methyl m-hydroxybenzoate (25 g., 0.16 mole) and finally methyl iodide (25 ml., 38 g., 0.27 mole). This solution was refluxed 5 hours, then cooled. A 5 N aqueous sodium hydroxide solution (5 ml.) was added and the solution extracted with diethyl ether (175 ml.). The diethyl ether was removed under vacuum and the residue refluxed 8 hours with 2.5 N aqueous sodium hydroxide (100 ml.). This solution was made strongly acid with concentrated hydrochloric acid and 16 g. (61%) m-methoxybenzoic acid was collected. The product, after one water crystallization, melted at 107-108°. Melting points of 105-106° and 106° have been reported for this compound (124, 125). This preparation is similar to that of Pierce et al. (126).

m-Methoxyhippuric Acid

An adaptation of the method of Acheson et al. (122) was used to synthesize this compound. Glycine (19 g., 0.25 mole) was dissolved in 0.5 N aqueous sodium hydroxide (80 ml.) and to it a solution of m-methoxybenzoyl chloride in diethyl ether (24 ml.) was added. The acyl chloride was made by refluxing m-methoxybenzoic acid (7.8 g., 0.051 mole) in thionyl chloride (10 ml.). After removal of the excess thionyl chloride under reduced pressure, the acyl chloride was dissolved in ether and used without further purification. A yield of 6.9 g. (53%) of m-methoxyhippuric acid was obtained. After crystallization from water, the compound melted at

123-125°. A melting point of 120° has been reported for this compound (123).

Methyl m-methoxyhippurate

m-Methoxyhippuric acid (6.5 g., 0.030 mole) was esterified with thionyl chloride (3.9 g., 0.033 mole) and methanol (70 ml.). A yield of 5.8 g. (87%) was obtained. The purified material melted at 78-79°.

Analysis:	calculated	C: 59.2	H: 5.9	N: 6.3
$C_{11}H_{13}NO_4$ (223)	found	C: 59.4	H: 6.0	N: 6.2

p-Ethoxybenzoic Acid

The method used for the synthesis of this compound was analogous to that for m-methoxybenzoic acid. The quantities of material used were the same except that the methyl iodide was replaced by ethyl iodide (20 ml., 30 g., 0.19 g.) and the methyl m-hydroxybenzoate was replaced by its para-substituted isomer. The yield was 29 g. (90%) of p-ethoxybenzoic acid, which melted at 196-197°, after crystallization from water. Jones and Robinson report a m. p. of 194° for this compound (125).

p-Ethoxyhippuric Acid

The method of Acheson et al. (122) was applied to the synthesis of this compound. As before, the acyl chloride was produced by a two-hour reflux of p-ethoxybenzoic acid (8.3 g.,

0.050 mole) in thionyl chloride (10 ml.). The same mole ratio of acyl chloride to glycine was used in this case as with m-methoxyhippuric acid. A yield of 6.8 g. (62%) of p-ethoxyhippuric acid was obtained. After crystallization from water, the compound melted at 154-155°.

Methyl p-ethoxyhippurate

Esterification of p-ethoxyhippuric acid (4.8 g., 0.022 mole) by the Brenner-Huber (103) method with thionyl chloride (3.3 g., 0.028 mole) and methanol (30 ml.) gave 4.6 g. (90%) of the methyl ester. Methyl p-ethoxyhippurate melted at 91-92° after four crystallizations from isopropyl ether.

Analysis:	calculated	C: 60.8	H: 6.4	N: 5.9
$C_{12}H_{15}NO_4$ (237)	found	C: 60.8	H: 6.2	N: 5.9

m-Ethoxybenzoic Acid

The procedure used in the synthesis of p-ethoxybenzoic acid was used to synthesize this compound, with the same mole ratios of reactants. The yield from 25 g. (0.16 mole) methyl m-hydroxybenzoate was 18 g. (66%) m-ethoxybenzoic acid, which melted at 136-137°, after crystallization from water. Pierce et al. (126) report a melting point of 131-135° for this compound.

m-Ethoxyhippuric Acid

The same procedure and the same molar ratios of reactants used to synthesize p-ethoxyhippuric acid were used to synthesize m-ethoxyhippuric acid. Treatment of glycine (19 g., 0.25 mole) with the acyl chloride made from m-ethoxybenzoic acid (8.3 g., 0.050 mole) gave 7.0 g. (64%) of m-ethoxyhippuric acid. This product melted at 150-151°, after crystallization from water.

Methyl m-ethoxyhippurate

Treatment of m-ethoxyhippuric acid (6.5 g., 0.029 mole) with thionyl chloride (4.0 g., 0.034 mole) and methanol (30 ml.) in the usual Brenner-Huber (103) esterification gave 6.2 g. (89%) of the ester. The purified ester melted at 70-71°, after four crystallizations from isopropyl ester.

Analysis:	calculated	C: 60.8	H: 6.4	N: 5.9
$C_{12}H_{15}NO_4$ (237)	found	C: 61.1	H: 6.2	N: 5.9

p-Isopropoxybenzoic Acid

The procedure used for the synthesis of this compound was an adaptation of the synthesis of p-ethoxybenzoic acid. The alkylation of methyl p-hydroxybenzoate (25 g., 0.16 mole) with redistilled isopropyl iodide (30 ml., 45 g., 0.27 mole) in a solution of sodium (6.0 g., 0.26 mole) in ethanol (90 ml.) gave p-isopropoxybenzoic acid in an 83% yield. This product melted at 162-163°, after crystallization from water. Pierce et al. (126) report a m.p. of 163-165° for this compound.

p-Isopropoxyhippuric Acid

The same method and the same mole ratios of starting materials were used in the synthesis of this compound as were used in the synthesis of p-ethoxyhippuric acid, except that p-ethoxybenzoic acid was replaced by p-isopropoxybenzoic acid (9.0 g., 0.050 mole). The product was obtained in 8.2 g. (69%) yield and melted at 137-138°, after crystallization from water.

Methyl p-isopropoxyhippurate

Thionyl chloride (4.4 g., 0.037 mole) and methanol (50 ml.) were used to prepare the methyl ester from p-isopropoxyhippuric acid (7.8 g., 0.033 mole) in a Brenner-Huber (103) esterification. After four crystallizations from isopropyl ether, the product melted at 81-82°.

Analysis:	calculated	C: 62.1	H: 6.8	N: 5.6
$C_{13}H_{17}NO_4$ (251)	found	C: 62.1	H: 6.9	N: 5.6

Methyl p-aminohippurate

p-Aminohippuric acid (19 g., 0.10 mole) was esterified with thionyl chloride (14 g., 0.12 mole) and methanol (100 ml.) by the procedure described previously. A yield of 17 g. (81%) of the ester was obtained. The product was crystallized four times from ethyl acetate and melted at 131-132°. Wolf and Niemann (18a) report 132-133° for the m.p. of this compound. A saponification equivalent of 206 ± 2 was determined for this compound, whose molecular weight is 208.2.

Ethyl p-methoxycinnamate

Technical grade ethyl acetate (300 ml.) was purified by a procedure described by Marvel and King (127). This purified ethyl acetate (150 ml., 206 g., 2.34 moles) was poured over finely divided sodium (10 g., 0.43 mole). A catalytic amount of dry ethanol (0.5 ml.) was added to bring the mixture into solution. After the solution had been cooled to 0^{o}, anisaldehyde (36 g., 0.26 mole) was added, with stirring and cooling, at such a rate that the temperature remained below 5^{o}. After all the anisaldehyde had been added, the solution was stirred an hour at 0^{o}. The solution was washed with dilute (5%) aqueous sodium bicarbonate, N aqueous hydrochloric acid and saturated aqueous sodium chloride, then dried over anhydrous magnesium sulfate. Ethyl p-methoxycinnamate, which melted at $47-51^{o}$, was obtained in a 30 g. (56%) yield, after removal of the ethyl acetate solvent under reduced pressure. Bowden and Adkins (128), who prepared the compound by a similar method, report a m. p. of $48-50^{o}$.

p-Methoxycinnamic Acid

Saponification of the ester just described (30 g., 0.14 mole) with 4 N aqueous sodium hydroxide (100 ml.) gave 25 g. (95%) of p-methoxycinnamic acid, which melted at $165-167^{o}$ (crude). It has been reported that the pure material melts at $173-174^{o}$ (129).

p-Methoxyhydrocinnamic Acid

A suspension of p-methoxycinnamic acid (25 g., 0.14 mole) in water (200 ml.) was reduced with an amalgam made of sodium (16 g., 0.70 mole) in mercury (550 g.). The mixture was stirred until a negative test for unsaturation with potassium permanganate solution was obtained (8.5 hours). The two phases were separated and the alkaline aqueous phase was treated with charcoal, filtered and acidified to give 22 g. (88%) of p-methoxyhydrocinnamic acid. This compound, purified by crystallization from water, melted at 101-104°. Walter et al. report a m.p. of 104-105° for this compound (130).

p-Hydroxyhydrocinnamic Acid

A solution of p-methoxyhydrocinnamic acid (11 g., 0.061 mole) in glacial acetic acid (75 ml.) was poured into hydriodic acid (34 ml. of 57% hydriodic acid, 0.26 mole). The mixture was heated at 85° for one hour, then refluxed 16 hours. The residue which remained after removal of the solvents was dissolved in 20 ml. of 0.5 N aqueous sodium hydroxide. The alkaline aqueous solution was extracted with diethyl ether, treated with charcoal and filtered. Acidification of the filtrate with concentrated hydrochloric acid gave 7.5 g. (75%) of p-hydroxyhydrocinnamic acid, which melted at 124-127°. Bowdin and Adkins (128) report that the pure compound melts at 128-129°.

Methyl p-hydroxyhydrocinnamate

A solution of p-hydroxyhydrocinnamic acid (7.5 g., 0.045 mole) in methanol (150 ml.) containing a catalytic amount of concentrated sulfuric acid (0.4 ml.) was refluxed for 11 hours. The solution was cooled and 25 ml. of water was added. This mixture was extracted with diethyl ether. The ethereal solution was washed with dilute (5%) aqueous sodium bicarbonate, N aqueous hydrochloric acid and saturated aqueous sodium chloride. After it had been dried over anhydrous magnesium sulfate, treated with charcoal and filtered, the ethereal solution was evaporated to dryness. The yield of crude product was 6.0 g. (74%). This material was distilled twice at 10 mm. Hg, b.p. 168-171°. The purified methyl p-hydroxyhydrocinnamate solidified on standing and melted at 38-40°. It has been reported that this compound melts at 40-41° (131). A saponification equivalent of 180 ± 5 was determined for this compound, which has a molecular weight of 180.1.

Kinetic Studies

The kinetic parameters of the α-chymotrypsin-catalyzed hydrolyses of the substrates in the present study were evaluated by a procedure which has been described previously (108, 132). All the kinetic determinations were conducted in aqueous solutions 0.10 M in sodium chloride, at 25.0° and pH 7.90 ± 0.10. The volume of the reaction solutions was 10 ml. and was contained in 20 ml. beakers which had an average inside diameter of 2.1-2.3 cm.

Bovine, salt-free α-chymotrypsin was used in all cases. In the experiments with the O-alkyl ethers of N-acetyl-L-tyrosine methyl ester and with N-methyl-N-acetyl-L-tyrosine methyl ester, Armour lot T-97209 α-chymotrypsin, containing 14.6% protein-nitrogen, was used. In all other cases, Worthington lot CDI6066-67 α-chymotrypsin, containing 14.9% protein-nitrogen, was used. For substrates requiring an enzyme concentration of 5.5×10^{-7} M or less, the enzyme stock solution was injected into the reaction solution by means of a syringe delivering 0.994×10^{-2} ml. The syringe was rinsed 5-10 times with the stock solution before each delivery. In all other cases, a syringe delivering 1.00 ml. was used. In the cases in which a 1.00 ml. volume of enzyme stock was used, the pH of the enzyme stock solution was adjusted to pH 7.9 before it was injected into the reaction solution. Adjustment of the pH of the more dilute enzyme solutions before addition to the reaction solution was found to be unnecessary.

The primary data consisted of automatically recorded traces of the rates of production of acid from the α-chymotrypsin-catalyzed hydrolyses at various concentrations of the substrates. The data were corrected for any detectable non-enzyme-catalyzed hydrolysis of the substrates. These data were analyzed on a Datatron 220 digital computer, programmed as described previously (35).

APPENDICES

Appendix 1

In well stirred solutions containing a species which can be adsorbed out of the solution onto a solid surface in contact with it, the solid is covered by a thin undisturbed layer of the solution (38, 39). The more efficiently the solution is stirred, the closer this layer approaches a minimum thickness. For aqueous solutions, the lower limit of this thickness is 3.0×10^{-3} cm. (38).

If the adsorption process is very rapid, the rate of diffusion of the adsorbed material through the immobile layer is rate limiting. This diffusion rate may be expressed (38, 39) as

$$V = \frac{\Delta n}{\Delta t} = \frac{DSc}{d} \qquad 4$$

where Δn is the amount of material adsorbed on the surface in an increment of time Δt, D is the diffusion coefficient of the material in the given solvent system, S is the surface area available for adsorption, c is the concentration of the material in solution, and d is the thickness of the immobile layer.

The validity of equation 4 depends on the constancy of D, of S, and of c (38, 39). It also is valid only if the concentration gradient across the immobile layer is linear and the concentration of material at the solid phase is negligible small compared to c. If the stirring of the solution is efficient, the concentration in solution c is uniform.

The diffusion coefficients of proteins are independent of the type and concentration of added electrolyte species (38, 41), provided the concentrations of added electrolyte are high enough and that the particular electrolyte has no effect on the protein. Trurnit measured the rates of adsorption of α-chymotrypsin on microscope slides in aqueous sodium veronal (sodium 5, 5-diethylbarbiturate) solutions at enzyme concentrations of 0.4 to 6.0×10^{-7}M (38). Variation of sodium veronal concentration from 10^{-3}M to 2×10^{-2}M and of the solution pH from 7.6 to 8.1 did not change the adsorption rate (38). Creeth found that variation of ionic strengths from 0.05 M to 0.20 M had no effect on diffusion rates of proteins in water (41). It has been reported that there is no special interaction between sodium veronal and proteins generally (41) nor α-chymotrypsin in particular (38). It will be assumed that the solvent conditions in Trurnit's experiments (38)- (0.01 M aqueous sodium veronal at pH 8.1)- are essentially identical to those reported in this thesis (0.10 M aqueous sodium chloride, pH 7.9), as regards the diffusion and adsorption process.

Trurnit measured the energy of activation for the diffusion of α-chymotrypsin as 5.1 ± 0.2 k. cal./mole (38). The adsorption rate at 25° is thus 1.2 times that at 20°, the temperature at which the reported adsorption measurements were made.

In Trurnit's experiments, the area of the protein-adsorbing surface and the solution volumes were such that the assumptions of constant surface area, constant protein concentrations in solution, and zero concentration of enzyme at the protein-adsorbing surface were valid for initial enzyme concentrations lower than 8×10^{-8}M and for time intervals of five minutes or more (38).

Below initial enzyme concentrations of 6×10^{-7}M, α-chymotrypsin forms a monolayer with a density of 4.8×10^{-4} mg. protein/cm.^{2} (38), on glass surfaces. The kinetic determinations reported in this thesis were carried out in solutions 10 ml. in volume contained in 20 ml. glass beakers. The inside diameters of these beakers are 2.1 to 2.3 cm., giving about 20 to 24 cm.^{2} surface available for protein adsorption. The maximum adsorption of the enzyme on this surface is 1.2×10^{-2} mg., or a concentration decrease from a solution 10 ml. in volume of 4.8×10^{-8}M. Decreases in the enzyme concentration of this magnitude suggest that the decrease in concentration of enzyme in solution and the decrease in surface available for adsorption should affect the adsorption rate quantitatively.

The calculations summarized in Tables XII through XVIII are estimates of the decrease in enzyme concentration from adsorption on the glass reaction vessels. In these calculations, it was assumed that the adsorption rates were constant over half-minute intervals. Corrections for the decrease in concentrations in solution and the decrease in adsorbing surface area were applied, then a new rate was computed after each half-minute interval. These calculations were performed for several initial enzyme concentrations. The estimated losses of enzyme from solution tend to be high, because the rates probably deviate (in a negative sense) from linearity over the half-minute time interval and because the estimates of available adsorbing surface area are high. These calculations do indicate how serious are the losses in enzyme concentration at the concentrations summarized in Table II of the Results section.

TABLE XII. $([E]_o)_o = 1.0 \times 10^{-8}M$

t, min	v, M/min. x 10^{10}	$\frac{([E]_o)_t}{([E]_o)_o}$, %	St/So, %
0.0	5.0	100	100
0.5	4.9	98	100
1.0	4.7	95	99
1.5	4.6	93	98
2.0	4.4	90	98
2.5	4.3	88	97
3.0	4.2	86	97
3.5	4.1	84	97
4.0	3.9	82	96
4.5	3.8	80	96
5.0	3.7	78	96
5.5	3.6	76	95
6.0	3.5	74	95
6.5	3.4	72	94
7.0	3.3	71	94
7.5	3.2	69	94
8.0	3.2	68	93

TABLE XIII. $([E]_o)_o = 2.0 \times 10^{-8}$M

t, min.	v, M/min. x 10^{10}	$\frac{([E]_o)_t}{([E]_o)_o}$, %	St/So, %
0.0	10.0	100	100
0.5	9.8	98	99
1.0	9.4	96	98
1.5	9.0	93	97
2.0	8.7	91	96
2.5	8.5	89	96
3.0	8.3	87	95
3.5	8.0	85	94
4.0	7.7	83	93
4.5	7.4	81	92
5.0	7.2	79	92
5.5	7.0	77	91
6.0	6.8	76	90
6.5	6.6	74	90
7.0	6.4	72	89
7.5	6.2	71	88
8.0	6.2	70	88

TABLE XIV. $([E]_o)_o = 4.0 \times 10^{-8}$M

t, min.	v, M/min. x 10^9	$\frac{([E]_o)_t}{([E]_o)_o}$, %	St/So, %
0.0	2.00	100	100
0.5	1.92	98	98
1.0	1.82	95	96
1.5	1.75	93	94
2.0	1.66	90	92
2.5	1.60	88	91
3.0	1.51	86	88
3.5	1.46	84	87
4.0	1.39	82	85
4.5	1.34	80	84
5.0	1.29	78	83
5.5	1.25	77	81
6.0	1.20	75	80
6.5	1.17	74	79
7.0	1.12	72	78
7.5	1.09	71	77
8.0	1.05	69	76

TABLE XV. $([E]_o)_o = 8.0 \times 10^{-8}$M

t, min.	v, M/min. x 10^9	$\frac{([E]_o)_t}{([E]_o)_o}$, %	St/So, %
0.0	4.00	100	100
0.5	3.76	98	96
1.0	3.50	95	92
1.5	3.31	93	89
2.0	3.09	91	85
2.5	2.92	89	82
3.0	2.75	87	80
3.5	2.62	85	77
4.0	2.49	84	74
4.5	2.36	82	72
5.0	2.24	81	69
5.5	2.14	80	67
6.0	2.03	78	65
6.5	1.94	77	63
7.0	1.85	76	61
7.5	1.75	74	59
8.0	1.66	73	57

TABLE XVI. $([E]_o)_o = 16 \times 10^{-8}M$

t, min.	v, M/min. x 10^9	$\frac{([E]_o)_t}{([E]_o)_o}$; %	St/So, %
0.0	8.00	100	100
0.5	7.21	98	92
1.0	6.46	95	85
1.5	5.80	93	78
2.0	5.31	91	73
2.5	4.77	89	67
3.0	4.44	88	63
3.5	4.04	87	58
4.0	3.67	85	54
4.5	3.43	84	51
5.0	3.12	83	47
5.5	2.89	82	44
6.0	2.66	81	41
6.5	2.43	80	38
7.0	2.28	79	36
7.5	2.15	79	34
8.0	2.00	78	32

TABLE XVII. $([E]_o) = 32 \times 10^{-8}M$

t, min.	v, M/min. x 10^8	$\frac{([E]_o)_t}{([E]_o)_o}$; %	St/So, %
0.0	1.60	100	100
0.5	1.32	98	84
1.0	1.08	95	71
1.5	0.90	94	60
2.0	0.75	92	51
2.5	0.63	91	43
3.0	0.53	90	37
3.5	0.46	89	32
4.0	0.38	88	27
4.5	0.34	88	23
5.0	0.28	87	20
5.5	0.24	87	17
6.0	0.21	87	15
6.5	0.18	86	13
7.0	0.15	86	11
7.5	0.13	86	9.2
8.0	0.11	85	8.0

TABLE XVIII. $([E]_o)_o = 96 \times 10^{-8}M$

t, min.	v, M/min. x 10^8	$\frac{([E]_o)_t}{([E]_o)_o}$, %	St/So, %
0.0	4.80	100	100
0.5	2.42	97	52
1.0	1.29	96	28
1.5	0.69	96	15
2.0	0.35	95	7.8
2.5	0.19	95	4.2
3.0	0.11	95	2.2
3.5	0.06	95	1.1
4.0	0.03	95	0.5
4.5	0.01	95	0.2
5.0	0.00	95	0.1
5.5	0.00	95	0.0
6.0	0.00	95	0.0
6.5	0.00	95	0.0
7.0	0.00	95	0.0
7.5	0.00	95	0.0
8.0	0.00	95	0.0

Trurnit found that as long as the adsorption rate was constant, the rate of change of the average thickness of the monolayer with time was 1.46×10^{6} angstroms/min./g. protein/ml. (38). The average thickness of the monolayer was measured as 35 angstroms and the average density of the monolayer was measured as 4.8×10^{-4} mg. protein/cm.^{2} (38). The ratio D/d of equation 4 is, therefore, 2.0×10^{-2} cm./min. Assuming a surface area of 25 cm.^{2}, equation 4 becomes

$$Vo = 5.0 \times 10^{-2} \text{ min}^{-1} c_o \qquad 5$$

where c_o is the initial concentration of α-chymotrypsin, in M concentration units. Equation 5 and its modifications described in the previous paragraph were used for computation of Tables XII through XVIII.

These calculations indicate that the decrease in enzyme concentration over the eight-minute time interval in which kinetic runs were made never exceeds 32% of the initial enzyme concentration. The decrease in enzyme concentration over the first two-minute period of the kinetic run never exceeds 10%. As mentioned previously, these estimates are probably high.

Appendix 2

Rotations of derivatives of N-acetyl-D-and-L-phenylalanine methyl ester[a]

Derivative	$[\alpha]_D$ of L	$[\alpha]_D$ of D
ortho-Fluoro	$+(10.5 \pm 0.4)^o$	$-(10.1 \pm 0.4)^o$
meta-Fluoro	$+(13.0 \pm 0.2)^o$	$-(12.1 \pm 0.4)^o$
para-Fluoro	$+(13.4 \pm 0.2)^o$	$-(10.3 \pm 0.3)^o$
ortho-Chloro	$-(18.6 \pm 0.5)^o$	$+(18.1 \pm 0.7)^o$
meta-Chloro	$+(15.0 \pm 0.3)^o$	$-(16.8 \pm 0.3)^o$
para-Chloro[b]	$+(25.2 \pm 0.4)^o$	$-(12.4 \pm 0.2)^o$
meta-Nitro	$+(11.0 \pm 0.3)^o$	$-(11.1 \pm 0.2)^o$
ortho-Hydroxy	$-(7.7 \pm 0.2)^o$	$+(7.8 \pm 0.2)^o$
ortho-Methoxy	$-(4.7 \pm 0.2)^o$	$+(4.3 \pm 0.2)^o$
meta-Hydroxy	$+(16.0 \pm 0.2)^o$	$-(13.5 \pm 0.2)^o$
meta-Methoxy	$+(17.2 \pm 0.5)^o$	$-(17.1 \pm 0.2)^o$
3,5-Dimethoxy	$+(18.0 \pm 0.5)^o$	$-(14.5 \pm 0.4)^o$
meta-Methyl	$+(20.7 \pm 0.4)^o$	$-(19.4 \pm 0.5)^o$
para-Methyl	$+(23.3 \pm 0.4)^o$	$-(20.7 \pm 0.3)^o$

a) Rotations were measured in methanol at concentrations of 1 to 3% and at temperatures of 22 to 28°. See the Experimental section for details.

b) Resolution stopped at 79% hydrolysis of one antipode.

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PROPOSITIONS

PROPOSITION I

Riordan and Vallee (1) have suggested that carboxypeptidase A catalyzes the hydrolyses of its amide substrates and its ester substrates by two different mechanisms. The authors (1) based this suggested difference in mechanism on the observation that the shapes of the pH-rate profiles for the enzyme-catalyzed hydrolysis of O-hippuryl-DL- β-phenyllactic acid and of N-carbobenzoxyglycyl-L-phenylalanine were different. Snoke and Neurath (2) had previously presented evidence which indicates that the carboxypeptidase A-catalyzed hydrolysis of this ester involves inhibition by excess substrate.

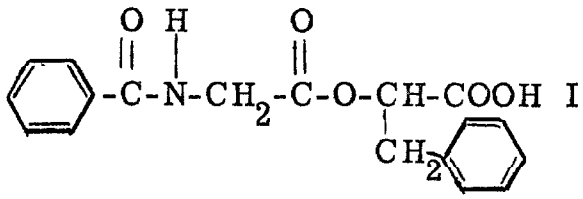
It is proposed that the observation of Riordan and Vallee (1) may be interpreted as a consequence of inhibition by excess substrate and not a difference in mechanism of action of the enzyme on the two classes of substrates. Experiments are proposed to test this interpretation.

The substrates of carboxypeptidase A have a free carboxyl group and a hydrolyzable amide or ester bond α to the carboxyl group (3-5). All the known substrates of this enzyme, including peptides, acylated α-amino acids and acylated α-hydroxy acids, are of the L configuration (3-5).

Usually, the rates of carboxypeptidase A-catalyzed hydrolyses in aqueous solutions at pH 7.5 and 25° conform to the Michaelis-Menten-Henri rate law (equation 1).

$$V = \frac{k_o [E]_o [S]}{K_o + [S]} \qquad 1$$

However, Snoke and Neurath (2) observed that the rates of the carboxypeptidase A-catalyzed hydrolysis of O-hippuryl-DL-β-phenyllactic acid (I) at pH 7.5 and 25° did not conform



I

to equation 1. The substrate concentration of I was varied from 10^{-3}M to 10^{-2}M (2). At these concentrations, the dependence of the enzyme-catalyzed rate on substrate concentration indicates inhibition by excess substrate (2). The racemate of I was used in these experiments, but Snoke and Neurath (2) showed that the L isomer was the substrate and that the D isomer did not inhibit its hydrolysis.

The steady-state rate equation derived from the following scheme (6, 7) is consistent with the kinetics observed for inhibition by excess substrate:

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

$$ES + S \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES_2 \xrightarrow{k_4} ES + P$$

where k_4 is less than k_2. The derived rate expression is given by equation 2:

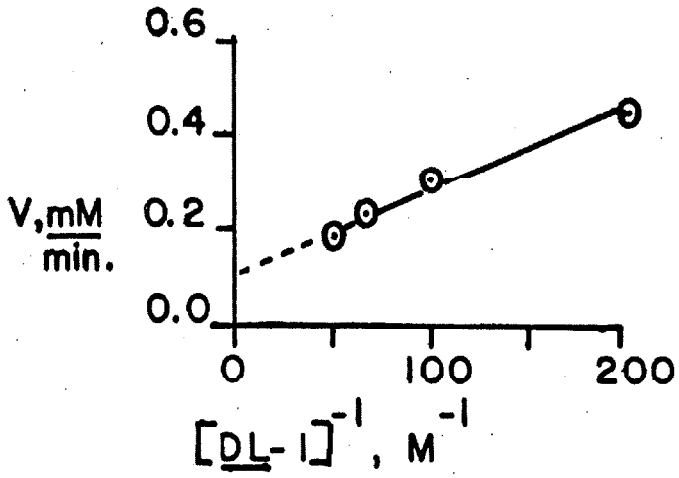
$$V = \frac{(k_2 K_{s2} + k_4 [S])\ [E]_o [S]}{K_{s1} K_{s2} + K_{s2} [S] + [S]^2} \qquad 2$$

where $K_{s1} = (k_{-1} + k_2)/k_1$ and $K_{s2} = (k_{-3} + k_4)/k_3$. If the magnitudes of K_{s1} and K_{s2} are much less than $[S]$, equation 2 reduces to equation 3:

$$V = k_4 [E]_o + \frac{k_2 K_{s2} [E]_o}{[S]} \qquad 3$$

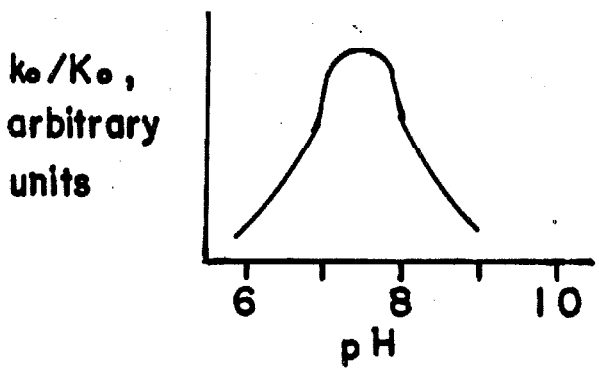
A plot of the rates of the carboxypeptidase A-catalyzed hydrolysis of I reported by Snoke and Neurath (2) as a function of the reciprocal of the concentration of the racemate of I shows that the data are consistent with equation 3 (see Figure 1).

Riordan and Vallee(1) report the pH-rate profiles of the carboxypeptidase A-catalyzed hydrolysis of N-carbobenzoxyglycyl-L-phenylalanine (II) and of I. The rates of



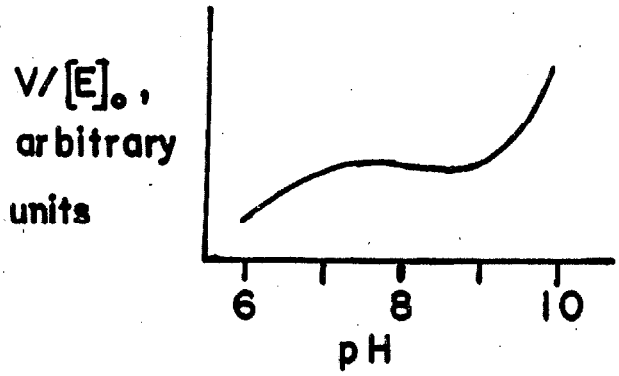
Conditions: aqueous solutions at pH 7.5, 25°; enzyme concentration constant at 0.25 μg. P-N/ml. (Data from ref.2.)

Figure 1. Carboxypeptidase A-catalyzed hydrolysis of DL-I.



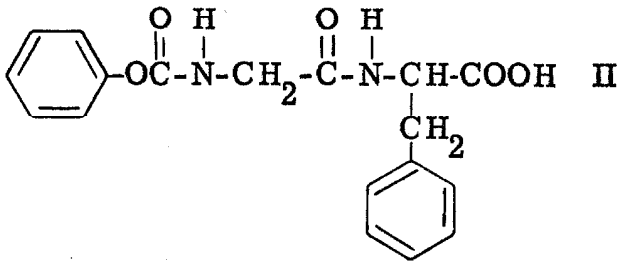
Type of pH dependence observed for the rates of the carboxypeptidase A-catalyzed hydrolysis of compounds II and III.

Figure 2. Bell-shaped pH-rate profile.



The pH dependence observed for the rates of the carboxypeptidase A-catalyzed hydrolysis of compound I. (See ref.1.)

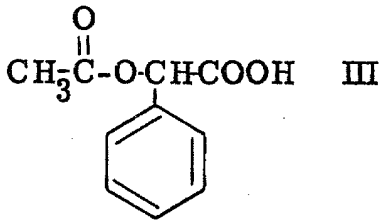
Figure 3. Sigmoid pH-rate profile.



II

the enzyme-catalyzed hydrolysis of II follow equation 1 (2). The plot of the ratio (ko/Ko) for II as a function of pH is bell-shaped with a maximum at pH 7.5 (1) (see Figure 2). The plot of the rates of the enzyme-catalyzed hydrolysis of I at 10^{-2}M in the racemate of I is sigmoid, with a plateau between pH 7 and 9 (1) (see Figure 3). Riordan and Vallee (1) concluded that the different shapes of these two curves indicate that the mechanisms of the carboxypeptidase A-catalyzed hydrolysis of the two compounds are different. Riordan and Vallee (1) and Vallee et al. (8) postulate that the enzyme-catalyzed hydrolysis of ester and amide substrates of carboxypeptidase A both occur at the same active site, but that one of the catalytic groups located there operates only on amides. This catalytic group is supposed to be replaced by hydroxide ion when esters are hydrolyzed (1, 8).

Kaiser and Carson (9) have recently published studies of the carboxypeptidase A-catalyzed hydrolysis of O-acetyl-L-mandelic acid (III). The rates of the enzyme-catalyzed



III

hydrolysis of III conform to equation 1 (9). The dependence of the ratio (ko/Ko) on pH is bell-shaped, with a maximum at pH 7.5 (9). This observation is inconsistent with the postulated mechanism for the carboxypeptidase A-catalyzed hydrolysis of its ester substrates given by Vallee and co-workers (1, 8). According to their mechanism, the pH-rate profile for an ester substrate should not be the same shape as that for an amide.

It is proposed that the pH-rate profile observed by Riordan and Vallee (1) for compound I is a result of substrate inhibition rather than an unusual bond-breaking mechanism. At the concentration of I for which the pH-profile was determined, equation 3 is obeyed. The pH profile of the rate of the enzyme-catalyzed hydrolysis of I is actually a function of the pH dependence of three kinetic constants and the combination of the three does not correspond to the ratio (ko/Ko). Therefore, no valid conclusions as to the mechanism of its enzymatic hydrolysis compared to the mechanism of the enzymatic hydrolyses of II and of III can be made.

The dependence of the rates of the enzymatic hydrolysis of I on the concentration of I indicate that equation 3, which is a special case of equation 2, is obeyed (2). From equation 2, one would predict that, as the substrate concentration is increased, the rate should initially be first order in substrate concentration, reach a maximum rate, then decrease and eventually approach a constant rate. In the first order region, the rate is given by equation 4:

$$V = \frac{k_2}{K_{s1}} [E]_o [S] \qquad 4$$

The ratio (k_2/K_{s1}) is comparable to the ratio (ko/Ko).

The maximum in the curve of the dependence of the rate of the enzyme-catalyzed hydrolysis of I as a function of substrate concentration has not been observed (2). However, a limiting minimum rate is approached at 10^{-2}M in I (2). Concentrations of I lower than 10^{-3}M have not been used and it is probable that at lower concentrations of I, the maximum rate and the first order region can be observed. (Often, in substrate-inhibited reactions, the transition from the first order region to the maximum rate is sharp (6).)

It is proposed that the carboxypeptidase A-catalyzed hydrolysis of compound I be studied at concentrations below 10^{-3}M in I. When the enzyme-catalyzed rate becomes first order in substrate concentration, the pH dependence of the first order constants (k_2/K_{s1}) should be determined. If the pH profile is bell-shaped with a maximum at pH 7.5, the mechanism of the carboxypeptidase A-catalyzed hydrolysis of I is the same as that for compounds II and III, and the sigmoid pH profile observed by Riordan and Vallee (1) is a result of substrate inhibition of the enzyme-catalyzed reaction. If the shape of the pH profile for I remains sigmoid, the mechanism of action of carbopeptidase A on compound I may be different from the mechanism of action of the enzyme on its other substrates. The results of Kaiser and Carson (9) show that the action of carboxypeptidase A on at least one of its ester substrates is similar to the action of the enzyme on its amide substrates.

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PROPOSITION II

The enzyme, nitrilase, catalyzes the conversion of nitriles to the corresponding carboxylic acids and ammonia (1-3). Mahadevan and Thimann (1) have postulated two covalent enzyme-substrate intermediates for the enzyme-catalyzed reaction. The first intermediate is a thioimidate ester formed by the reaction of the nitrile substrate with a thiol group on the enzyme. The second intermediate is an acyl-enzyme formed by the hydrolysis of the first intermediate.

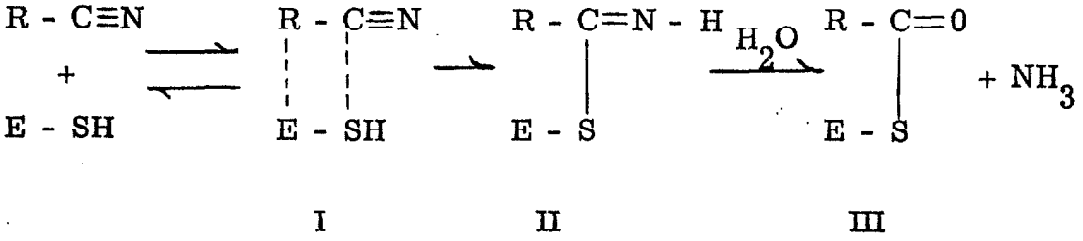
A method for detecting both of these postulated intermediates is proposed.

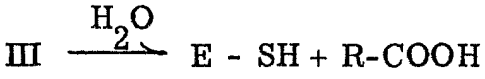
The enzyme, nitrilase, has been isolated by Mahadevan and Thimann (3) from the barley plant. The enzyme does not require metal ions or cofactors for full enzymatic activity (2). It is a hydrolytic and not an oxidative enzyme, since oxygen is not required for enzymatic activity (3).

Thiol reagents such as mercuric ions, cupric ions, N-ethylmaleimide and para-hydroxymercuribenzoic acid inactivate the enzyme (2). Glutathione can restore the enzymatic activity destroyed by para-hydroxymercuribenzoic acid (2). Thimann and Mahadevan (2) concluded that a thiol group is present in the enzyme and that it is essential for enzymatic reactivity.

Both alkyl and aryl cyanides can function as substrates of nitrilase (1). Thimann and Mahadevan (3) showed that the amides of the acids derived from the nitrile substrates are not substrates of nitrilase and that the amides are not intermediates in the enzyme-catalyzed reaction. Apparently the mechanism of the action of the enzyme on nitriles is different from the mechanism of the hydrolysis of nitriles in aqueous solutions (4).

On the basis of this, and other, evidence, Mahadevan and Thimann (1) proposed the following mechanism:





In this mechanistic scheme, E - SH is the sulfhydryl-bearing enzyme, I is the enzyme-substrate intermediate, II is a thioimidate ester intermediate, and III is an acyl-enzyme intermediate.

Imidate esters and thioimidate esters react with amines to form amidines (4-6). The formation of amidines by the attack of various amines on imidate esters has been studied in aqueous solutions by Jencks and Hand (6). The mechanism of the reaction is complex, but it is known that nucleophilic amines (such as

hydroxylamine) compete effectively enough with water to completely exclude the hydrolysis products at neutral pH's (6). The amination of thioimidate esters in aqueous solutions has not been studied, so that the relative nucleophilicities of hydroxylamine and water toward these compounds is unknown. However, hydroxylamine should be at least as good a nucleophile as water toward the thioimidate esters.

It is proposed that hydroxylamine be added to an aqueous solution of nitrilase and one of its substrates. Formation of the amidoxime derived from the nitrile substrate would support the postulated existence of the thioimidate ester intermediate (II).

If the acylated thiol intermediate III is formed in the nitrilase reaction, it too should react with hydroxylamine. Krimsky and Racker (8) were able to react hydroxylamine with the acylated thiol of glyceraldehyde-3-phosphate dehydrogenase (formed by reaction with para-nitrophenyl acetate (9)). The formation of the hydroxamate of the acid derived from the nitrile substrate would support the postulated intermediacy of acyl-nitrilase (III).

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PROPOSITION III

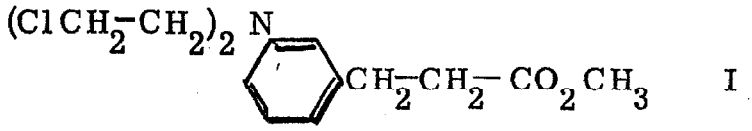
The nitrogen mustard meta-chlorambucil methyl ester is proposed as a potential irreversible inhibitor of α-chymotrypsin.

The use of selective reagents which react at, or near, the active site of α-chymotrypsin has made possible the identification of one serine residue (1, 2) and one histidine residue (3) as probable catalytic groups involved in the action of this enzyme. In addition, it has been shown that one methionine residue is probably involved in the process of binding of a substrate to this enzyme (4).

No reagent has yet been designed which might be expected to react at that portion of the active site of α-chymotrypsin where the side chains of substrates are bound (designated the ρ_2 locus (5)). The interaction of the substrate side chain with the ρ_2 locus is thought to contribute significantly to the effectiveness of the binding of the substrate to α-chymotrypsin (5). It is, therefore, of interest to attempt to identify one or more of the amino acids located at the ρ_2 locus.

The potential ρ_2 reagent must meet two requirements. First, the reagent should be bound to the active site in predominantly one mode, and that mode should maintain an interaction at ρ_2. Second, there should be some assurance that the reagent will form a covalent bond at ρ_2, once it is bound there.

A compound which meets these two requirements is the methyl ester of meta- [bis-(2-chloroethyl)amino]-hydrocinnamic acid (I) or meta-chlorambucil methyl ester (6). This compound should be bound to



I

α-chymotrypsin in predominantly one mode, and steric hindrance to binding in the ρ_2 locus should be less than for an isomer of I containing the substituent in an ortho or para position (7). The nitrogen mustards, which contain the bis-(2-chloroethyl)amino group, are known to alkylate the amino, carboxyl, imidazole, phenolic hydroxyl and sulfide groups of proteins (8). Among the amino acid residues of α-chymotrypsin which could be located at, or near, the ρ_2 locus of α-chymotrypsin and which could react with a nitrogen mustard are 14 lysines, 9 aspartic acids, 5 glutamic acids, one histidine, 5 tyrosines and one methionine (9). There are also three amino end groups and three carboxyl end groups (although one of the carboxyl end groups is a tyrosine residue (9)). There are 242 amino acid residues in the primary structure of α-chymotrypsin. There is thus some chance that compound I will react with an amino acid residue at, or near, the ρ_2 locus of α-chymotrypsin.

It is proposed that compound I be investigated as a potential irreversible inhibitor of α-chymotrypsin. If compound I attacks the enzyme indiscriminately, it could be modified by replacing the

chlorines on the mustard group with fluorines to make a more selective reagent.

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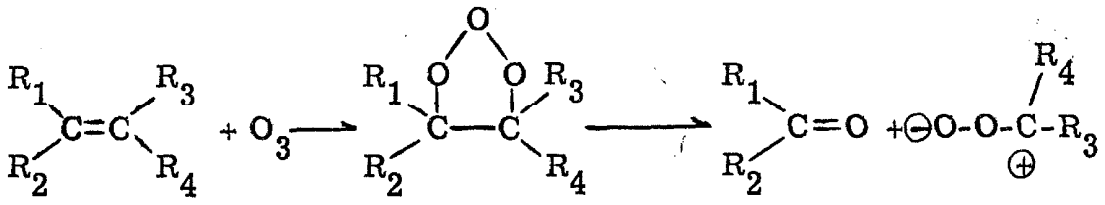
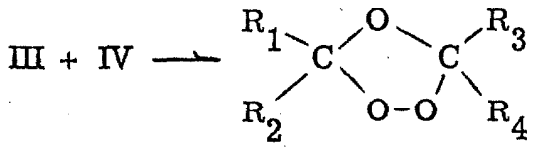
PROPOSITION IV

Cis-and trans-isomers of di-substituted ethylenes are attacked by ozone at different rates. For at least one pair of olefins, different products are formed from the two isomers. It is possible that two different mechanistic pathways exist for the ozonization of olefins. An experiment is proposed to test this possibility.

The currently accepted mechanism of ozonization of olefins is that proposed by Criegee (1, 2). According to this mechanism, ozone adds to the double bond of an olefin (I) to give an initial adduct (II) which decomposes into two fragments. One of the fragments is a carbonyl product (III) and the other fragment is a zwitterionic species (IV). The zwitterion can react with the carbonyl product to give the ozonide (V) or undergo several other reactions. The zwitterion (IV) is the key intermediate in this reaction.



I II III IV



V

The many products isolated from ozonization reactions can be explained in terms of the intermediate IV (1, 2). Evidence has been presented which supports its existence, in certain cases (1, 2).

Recently, Criegee and Schroder (3) and Greenwood (4) have reported evidence which supports the existence of intermediate II. However, it could be detected only in the ozonization of mono-substituted ethylenes and trans-di-substituted ethylenes (3, 4). In no case was an intermediate like II detected in the ozonization of cis-di-substituted ethylenes (3, 4). Apparently the "molozonides" (II) from cis- olefins are too unstable to be detected.

Generally, cis-di-substituted ethylenes are attacked by ozone more slowly than are their trans-isomers (1, 4). The instability of the intermediate II for the cis-olefins could explain this difference.

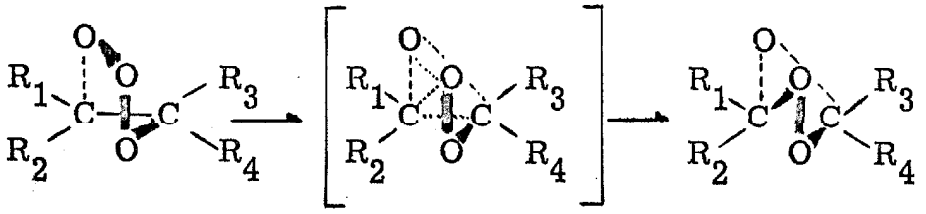
Schroder (5) has observed further differences in the reaction of ozone with cis-and trans-olefins. Cis-di-t-butylethylene (R_1, R_3 = H; R_2, R_4 = t-butyl in formula I) gives two isomeric ozonides (in a weight ratio of 3 to 7) upon attack by ozone, while in the same solvent (pentane) and at the same temperature (-75°) the same concentration of the trans-olefin gives only the isomeric ozonide present in lower concentration from ozonization of the cis-isomer. The combined yield of ozonides from the cis-olefin was 82%, compared to 58% its trans-isomer. The trans-olefin also gives a high yield of

peroxides. Schroder (5) assigned a trans-configuration to the ozonide from the trans-olefin.

The results from the ozonization of the trans-olefin are understandable in terms of the Criegee mechanism. If the fragments III and IV are produced, an ozonide with the cis-configuration would be difficult to form because it would require an addition of the zwitterion IV (R_3 = H, R_4 = t-butyl) to the carbonyl product (pivalaldehyde, in this case) which would be hindered by the bulky t-butyl groups. The high yield of peroxides from the ozonization of trans-di-t-butylethylene is also consistent with the production of the zwitterionic fragment, since polymerization of the zwitterion is the explanation given for the presence of peroxides among the ozonization reaction products (1, 2).

If the fragments III and IV are produced in the ozonization of cis-di-t-butylethylene, it is reasonable to expect that the same isomer of the ozonide should be produced, since the fragments would be identical to those produced from the trans-olefin. Furthermore, the the yield of peroxides should be the same for both olefins.

It is possible that the intermediate II could proceed to the ozonide V by a concerted collapse. The transition state may be somewhat



II V

like the one postulated for the decomposition of 9-decalyl peroxybenzoate (6). This concerted collapse should be possible for both molozonides of cis-and trans-di-t-butylethylene, but it may be the more important pathway for the cis-compound, in which steric repulsions between R_2 and R_4 would strain the five-membered ring of II.

Part of the evidence which supports the postulated existence of the zwitterionic species (IV) is the apparent exchange of a foreign aldehyde for the carbonyl compound III produced from the olefin (1, 2). If II rearranges to V by a concerted mechanism, no ozonide incorporating a foreign aldehyde should be produced.

It is proposed that cis-and trans-di-t-butylethylene be ozonized in pentane solutions containing carbon-14 labelled pivalaldehyde. This foreign aldehyde should be as reactive toward the zwitterion IV as the pivaldehyde produced by ozonization of the olefin. The yield of ozonide from the trans-olefin should be increased by inclusion of the pivalaldehyde in the reaction system and the yield of peroxides should be decreased. The presence of label in the trans-ozonide would show whether foreign aldehyde had been incorporated in the ozonide structure. The trans-ozonide from the cis-olefin should incorporate some carbon-14 label, but the cis-ozonide should not incorporate any carbon-14 label, if the concerted mechanism is applicable.

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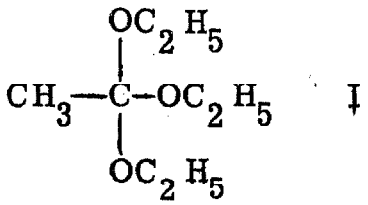
PROPOSITION V

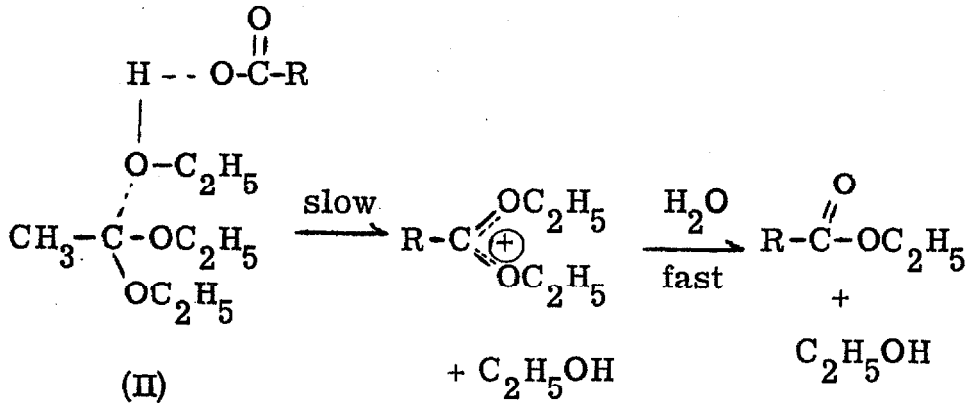
DeWolfe and Jensen (1) have concluded that the general acid catalyzed hydrolysis of ortho esters occurs by a unimolecular mechanism. These authors propose two possible transition states for the reaction mechanism. An experiment is proposed which should help determine which of the two transition states is the more probable one.

On the basis of thermodynamic data, isotope effects and substituents effects, DeWolfe and Jensen (1) concluded that the rate-determining step of the general acid catalyzed hydrolysis of triethyl orthoacetate (I) is a slow decomposition of a complex between I and the general acid. The authors



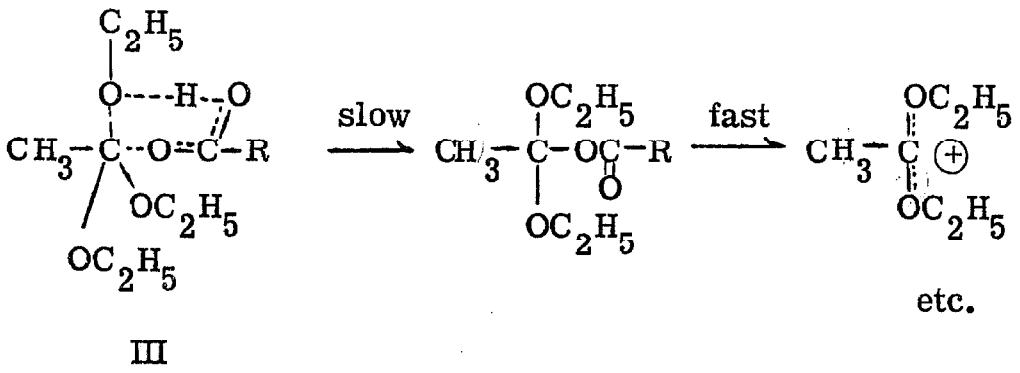
I

propose two possible transition states for this step. One transition state involves a hydrogen bonded complex between I and the general acid. (See II).



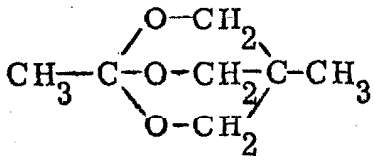
(II)

The second mechanistic pathway proposed by DeWolfe and Jensen (1) involves a cyclic complex between the ortho ester and the general acid (see III) which slowly decomposes to a dialkoxy acyl intermediate which, in turn, rapidly decomposes to the carboxonium ion.



III

It is proposed that the rate of the general acid catalyzed hydrolysis of 1, 4-dimethyl-2, 6, 7-trioxabicyclo [2. 2. 2.]octane (IV) be measured and compared to the rate of the general acid catalyzed hydrolysis of I. (This compound has been



IV

synthesized by Barnes et al. (2)). If the mechanism of ortho ester hydrolysis proceeds through transition state III, compound IV should be hydrolyzed much more slowly than compound I. If transition state II is applicable, compound IV should be more rapidly hydrolyzed than compound I. Access to the acyl carbon of IV is sterically hindered, but its oxygens are more exposed to possible attack by a general acid.

The possibility that the mechanisms of general acid catalyzed hydrolysis are different for I and IV could be checked by Leffler's criterion (3).

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