I. STERIC AND ELECTROSTATIC REPULSIONS IN THE INHIBITION OF α-CHYMOTRYPSIN CATALYSED HYDROLYSES BY INDOLE DERIVATIVES

II. STERIC REQUIREMENTS FOR SUBSTRATES OF α-CHYMOTRYPSIN

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Abstract

The enzyme-inhibitor dissociation constants, i.e., K_I's, were evaluated for the six isomeric pairs of C-substituted indolecarboxylate ions and carboxamides. The variation of K_I with the position and nature of the substituent indicates that the enzyme-indole complex exhibits a high degree of steric hindrance near the 4 position of the indole ring and electrostatic repulsion due to a negative group near the indole nitrogen.

The synthesis of D,L-β,β-dimethylphenylalanine was modified by use of air oxidation of 4,6-di-(α,α-dimethylbenzyl)pyrogallol to 3,5-di-(α,α-dimethylbenzyl)coumalic acid and permanganate oxidation of this product to obtain α-keto-β-phenylisovaleric acid. The by-products of the air oxidation were investigated.

D,L-2,6-Dimethyltyrosine, a previously unreported amino acid, and several of its derivatives were synthesized.

Studies on the rates of α-chymotrypsin catalysed hydrolyses of N-acetyl-D,L-t-leucine methyl ester, N-acetyl-D,L-β,β-dimethylphenylalanine methyl ester and N-acetyl-D,L-2,6-dimethyltyrosine methyl ester indicate the presence of a strong β steric effect.

Methods of resolution of D,L-β,β-dimethylphenylalanine and D,L-2,6-dimethyltyrosine derivatives were investigated.

Methyl indole-2-carboxylate is not a substrate of α-chymotrypsin.

Table of Contents

PART I

STERIC AND ELECTROSTATIC REPULSIONS IN THE INHIBITION OF α-CHYMOTRYPSIN CATALYSED HYDROLYSES BY INDOLE DERIVATIVES

Introduction:

The presence of coulombic repulsions toward anionic, competitive inhibitors by groups on, or near, the active site of α-chymotrypsin is a well established fact. A study of published values (1-17) of inhibition constants yields no case for which the inhibition constant of an anionic species is less than, or equal to, the inhibition constant of a structurally similar, neutral molecule. When comparisons of the inhibition constants, K_I's, are made for six pairs of carboxylate ions and their corresponding amides, it is seen that the ratio, $K_{I_{RCOO^-}}/K_{I_{RCONH_2}}$, at pH 7.9, varies from 3.3, in the case of α-N-acetyl-D-tryptophanate, α-N-acetyl-D-tryptophanamide (18), to 15, in the case of benzoate, benzamide (16). Furthermore, it has been shown, for several carboxylate ions (18), that the inhibition constants of anionic species decrease as pH is decreased from 7.9 to 6.9 while the inhibition constants of neutral analogues are unchanged over the same pH range. This clearly indicates the presence of an acidic group on, or near, the active site of the enzyme. As the nature of the observed repulsions of anionic inhibitors is coulombic and therefore subject to an inverse dependence on the distance between the negative group on the active site of the enzyme and the negative group of the inhibitor, a study of the variation of the inhibition constants of a series of structurally similar carboxylate ions would be useful in locating the position of the acidic group of the enzyme, relative to the common

structural features of the inhibitors. A similar study of the corresponding amides would distinguish between steric and electrostatic repulsions.

Since the suggestion (19) has been made that the active site of α-chymotrypsin shows a general affinity for binuclear aromatic systems, and since it is possible that inhibition would not be greatly affected by the orientation of a monofunctional, aromatic species, a study of the variation of inhibitor constants of the isomeric indole-carboxylate ions would be useful for determining whether a preferred orientation of the indole-α-chymotrypsin complex does exist. If no such preferred orientation exists, the complex would be formed in such a way as to minimize interaction between the negative groups and the inhibition constants of the indolecarboxylate ions would be independent of position of substitution. If a preferred orientation exists, marked variations in the inhibitor constants with position of substitution might be expected. Because of their generally low inhibition constants, high solubility and the complete lack of symmetry of the indole ring, indole derivatives are particularly useful in this sort of study. The availability of six carbon-substituted isomers affords a good opportunity for determining the directional features of the repulsions exerted by the active site of α-chymotrypsin towards competitive inhibitors.

Two acidic groups have been observed to be involved in α-chymotrypsin catalysed hydrolyses (20-23). The more acidic, with a pK_a determined to be between 6.7 (22) and 7.2 (23), is unaffected by substrate

binding but must be in the dissociated form in order that enzyme substrate complex decompose to form products. The second group, with a pK_a of 8.0 (23) to 8.6 (20), must be protonated for the enzyme to complex with substrate. It has not been firmly established which, if either, of these two groups is responsible for the repulsion of anionic inhibitors, although Gordon has suggested that it is the more basic group, on the basis of the pH dependence of the K_I's of hippurate and L-tryptophan. However, comparisons of effects observed for polyfunctional inhibitors (24), such as hippurate and L-tryptophan, with effects observed for indolecarboxylate ions, which should be considered monofunctional, are of a questionable nature. The question of assigning observed electrostatic repulsions to particular groups awaits more detailed pH dependence data and a more careful consideration of the relationship between the observed pH and the microscopic enzyme-inhibitor dissociation constant.

Summary:

The enzyme-inhibitor dissociation constants, i.e., K_I values, were determined for indole and its six pairs of C-substituted carboxylate ions and carboxamides at pH 7.60, 25.0° C, and a NaCl concentration of 0.20 M, using N-acetyl-L-tyrosinhydroxamide as a standard substrate. Where possible, two inhibitor concentrations were used, one of which was in the range $K_I < (I) < 3K_I$.

All of the compounds studied were found to be competitive inhibitors of α-chymotrypsin. None of the indolecarboxamides is a stronger inhibitor than indole and the carboxylate ions have K_I values at least twice as large as their corresponding amides. K_I values are highly dependent on position of substitution and the values for indole-4-carboxamide, indole-4-carboxylate ion and indole-7-carboxylate ion are particularly large with respect to the values of their isomers. This indicates maximum steric repulsion near the 4 position of indole and the presence of a negative charge near the indole nitrogen atom.

Experimental:

All inhibition constants were determined at pH 7.60, 25.0° C, and 0.2 M NaCl, using N-acetyl-L-tyrosinhydroxamide as the standard substrate. The advantages of this system are the fast linear kinetics of the substrate hydrolysis, the absence of blank reactions and the solubility of the substrate, making a wide range of substrate concentrations available. There is some disadvantage in the lower pH optimum of the substrate since comparison with literature values of K_I are less reliable, especially for charged inhibitors.

Materials:

Indole-2-carboxylic acid was prepared by the Johnson (25) modification of the Reissert(26) method and by the Rydon-Tweddle (27) modification of the Fischer (28) indole synthesis. The amide was prepared from the acid via the acid chloride (25). Indole-3-carboxylic acid and amide were prepared from the polymeric "indole-3-carbonyl chloride" of Peterson, Wolf and Niemann (29). The benzene substituted indole carboxylic acids were prepared by formation of the corresponding chloroindole-2-carboxylic acid, by the methods of Rydon and Tweddle (27) and of Uhle (30), formation of the corresponding nitriles by reaction with cuprous cyanide in quinoline (30,31), and hydrolyses to the acids. The amides were obtained by hydrolyses of the nitriles, using the procedure of Galat (32). Reagent grade indole (Matheson, Coleman and Bell) was recrystallized twice from water. Sodium N-acetyl-L-tyrosinhydroxamate

was prepared by a method similar to that of Hogness and Niemann (33).

o-Nitro-α-acetamidocinnamic Acid Azlactone: A mixture of o-nitrobenzaldehyde (9.7 g., 0.064 mole), aceturic acid (8.8 g., 0.075 mole), anhydrous sodium acetate (4.2 g., 0.051 mole), and of acetic anhydride (24 ml., 22.2 g., 0.195 mole) was warmed on a steam bath. The mixture turned red after fifteen minutes. Heating was continued for 3 hours and the mixture allowed to stand at room temperature overnight, during which time the reaction mixture crystallized. The solid mass was pulverized, transferred to a sintered glass filter, washed with five 20 ml. portions of water and dried over $CaCl_2$, in vacuo. A dark, yellow solid (9.5 g., 0.043 mole, 66% yield), smelling of acetic acid, m.p. 107-109° (Lit. 114-115°) (25) was obtained.

o-Nitrophenylpyruvic Acid: Crude o-nitro-α-acetamidocinnamic acid azlactone (9.5 g., 0.043 mole) was hydrolysed in 250 ml. of 1N HCl, at reflux, for 2.5 hours. The solution was treated with Norite and filtered while hot. A mixture of red oil, which slowly crystallized, and yellow platelets (1.2 g.) m.p. 109-112°, precipitated on cooling the filtrate. The mother liquor was extracted with five 30 ml. portions of ether and the combined extracts dried over Na_2SO_4 and evaporated to a red, oily residue. The residue was dissolved in 60 ml. of water and 25 ml. of saturated $NaHCO_3$ solution, and the solution acidified with HCl. A slowly crystallizing red oil (2.5 g.) and pale yellow platelets (2.1 g.), m.p. 115-117° (decomp.) (25) (Lit. 119-120°) precipitated. After the volume of the mother

liquor was reduced by half, tan crystals (0.9 g.) were obtained. Complete evaporation of the mother liquor and recrystallization of the residue from water gave additional product (1.1 g.). The total combined yield was 7.8 g. (0.040 mole, 92% yield).

Pyruvic Acid Phenylhydrazone: When a freshly filtered solution of phenylhydrazine hydrochloride (10.0 g., 0.069 mole) in 100 ml. of water was added to a solution of pyruvic acid (6.5 g., 0.074 mole) in 100 ml. of water, a copious yellow precipitate formed. It was collected and recrystallized from aqueous ethanol to obtain yellow needles (10.4 g., 0.058 mole, 84% yield), m.p. 180-182° (Lit. 192°) (34).

Ethyl Pyruvate Phenylhydrazone: Pyruvic acid phenylhydrazone (10.0 g., 0.056 mole) was suspended in 300 ml. of absolute ethanol. The mixture was chilled in a salt-ice bath and saturated with anhydrous HCl, after which all solid material had dissolved. The solution was allowed to stand overnight, evaporated, in vacuo, to 75 ml. and cooled. Colorless, hexagonal platelets (9.4 g., 0.046 mole, 82% yield), m.p. 112-115° (Lit. 117-118°) (34) crystallized and were collected.

Ethyl Indole-2-Carboxylate: Ethyl pyruvate phenylhydrazone (9.4 g., 0.046 mole) was mixed with polyphosphoric acid (20 g.) and the mixture heated. At 50° an exothermic reaction occurred, the temperature of the reaction rapidly rising to 130°. The reaction mixture was maintained at 160° for 20 minutes, then allowed to cool to 60°. One hundred ml. of water was mixed with the reaction mixture whereupon a yellow-brown solid formed, which was collected by filtration, washed

with water and dissolved in ether. The filtrate was washed three times with ether and the combined ether solutions were dried over Na_2SO_4 and evaporated, in vacuo, at 25°, to a yellow solid. Recrystallization of this residue from water gave product (3.25 g., 0.0172 mole, 37% yield), m.p. 116-121°; (Lit. 116-117°) (28).

Indole-2-carboxylic Acid: I. (From o-nitrophenylpyruvic acid) o-Nitrophenylpyruvic acid (7.8 g., 0.040 mole) was dissolved in 135 ml. of warm, 7N NH_4OH. A warm solution of $FeSO_4 \cdot 7H_2O$ (68 g., 0.20 mole) was gradually added to the above solution and the suspension that resulted was warmed on the steam bath for 30 minutes and then boiled for 30 minutes, in order to coagulate the precipitate. The mixture was filtered and the precipitate washed with 300 ml. of warm 5N NH_4OH. Considerable amounts of suspended precipitate passed through the filter but, on acidification with 100 ml. of 12N HCl, the suspended material dissolved and grey crystals formed. This precipitate was collected and dissolved in ether, the solution washed three times with 6N HCl, dried over Na_2SO_4, and evaporated to a grey solid. Recrystallization of the solid residue from benzene gave nearly white platelets (1.05 g., 0.0065 mole), m.p. 200-205° (decomp.). Additional yellow product (0.40 g., 0.0025 mole) was obtained by extraction of the acid washings with ether. The product was completely decolorized by boiling a slightly alkaline aqueous solution, containing a trace of hydroquinone, with Norite for one minute, filtering and careful acidification with HCl to obtain long colorless needles (0.85 g., 0.0053 mole, 13% yield), m.p. 200.5-202.0° (Lit. 202-204°)(25).

Analysis: Calculated for $C_9H_7NO_2$ (161): C: 67.07%; H: 4.38%; N: 8.69%

Found: C: 67.14%; H: 4.36%; N: 8.70%

II. (From ethyl indole-2-carboxylate) Ethyl indole-2-carboxylate (3.25 g., 0.0172 mole) was dissolved in a solution of KOH (15 g., 0.27 mole) in 160 ml. of 95% ethanol. The solution was heated at reflux for 4 hours, cooled and poured into 450 ml. of 1N HCl. The solution was evaporated, in vacuo, until the first crystallization occurred, and stored in the refrigerator overnight. Buff colored crystals (1.95 g., 0.0121 mole, 70% yield), m.p. 201-206° were collected by filtration.

Indole-2-carboxamide: Crude indole-2-carboxylic acid (1.67 g., 0.0103 mole) was dissolved in 60 ml. of anhydrous ether and the solution chilled in a salt-ice bath. Thionyl chloride (1.55 ml., 2.54 g., 0.0214 mole), freshly distilled over quinoline and raw linseed oil, was added and the solution allowed to stand in the ice bath for 45 minutes and at room temperature for thirty minutes. It was then evaporated, in vacuo, below 30°, nearly to dryness. About 15 ml. of absolute ether was added and the evaporation repeated. The solution and evaporation cycle was repeated a third time, the residue was dissolved in 50 ml. of anhydrous ether, and the solution added to 50 ml. of a cold, ammonia saturated ether solution and anhydrous ammonia passed through the mixture for an additional 15 minutes. Ammonium chloride was removed by filtration and the filtrate evaporated to dryness. The ammonium chloride precipitate was washed with 75 ml. of methanol and the washings used to dissolve the residue of the ether filtrate. The methanol solution was concentrated

by evaporation and adjusted, while warm, to the cloud point by addition of water. Tan platelets (0.96 g., 0.0060 mole) crystallized. Recrystallization from water, with treatment with Norite, gave colorless granules (0.35 g., 0.0022 mole, 21% yield), m.p. 234-235.5° (Lit. 234.5-235.5°) (35).

Analysis: Calculated for $C_9H_8N_2O$ (160): C: 67.50%; H: 5.03%; N: 17.49%

Found: C: 67.33%; H: 5.20%; N: 17.73%

Indole-3-glyoxalyl Chloride: Oxalyl chloride (6.5 ml., 9.7 g., 0.076 mole) was added to a cold solution of indole (10.0 g., 0.084 mole) in 100 ml. of cold, anhydrous ether. Yellow indole-3-glyoxalyl chloride precipitated immediately and was collected on sintered glass and dried by passing dry air through the material.

Polymeric "Indole-3-carbonyl Chloride": The crude indole-3-glyoxalyl chloride prepared above was dissolved in 100 ml. of s-tetrachloroethane and the solution maintained at 120° for two minutes, during which time the solution darkened and foamed vigorously. When the solution was cooled in a water bath and 300 ml. of hexane added, a dark brown precipitate of polymeric "indole-3-carbonyl chloride" formed. The material (9.6 g.) was collected and washed with hexane.

Indole-3-carboxylic Acid: Polymeric "indole-3-carbonyl chloride" (4.4 g.) was stirred in 100 ml. of 1N $NaHCO_3$ for 3 hours. The brown residue was removed by filtration and the filtrate saturated with nitrogen and acidified with HCl. The precipitate that formed, and which rapidly became intensely red on contact with its mother liquor, was

quickly centrifuged, washed twice with water and collected, under a nitrogen atmosphere, on sintered glass. Recrystallization of crude pink material from 10% aqueous acetone gave colorless needles, m.p. 235-239° (decomp.) (Lit. 240°) (36), whose inhibitor strength was independent of the age of the stock solution.

Analysis: Calculated for $C_9H_7NO_2$(161): C: 67.07%; H: 4.38%; N: 8.69%

Found: C: 66.96%; H: 4.51%; N: 8.64%

Indole-3-carboxamide: Polymeric "indole-3-carbonyl chloride" (7.5 g.) was added to 100 ml. of a chilled, ammonia saturated ether solution. The mixture was allowed to stand at room temperature for 30 minutes, the ammonium chloride precipitate was removed by filtration and the filtrate evaporated to dryness. Recrystallization of the residue gave a pale orange product. Further decolorizing could be achieved only by elution of a methanol solution of the material through a 13 cm. column of Norite A. The eluant was recrystallized twice from aqueous methanol to obtain large, colorless laminae (1.15 g., 0.0072 mole), m.p. 204-205.5° (Lit. 201°) (37).

Analysis: Calculated for $C_9H_8N_2O$ (160) C: 67.48%; H: 5.03%; N: 17.49%

Found: C: 67.37%; H: 5.37%; N: 17.56%

In several experiments, the major product was not indole-3-carboxamide but an unidentified product obtained in the form of colorless platelets, m.p. 122-123.5°.

Analysis: Calculated for C_7H_7NO (121) C: 69.40%; H: 5.83%; N: 11.56%

$C_{21}H_{19}N_3O_3$(361.4) C: 69.79%; H: 5.30%; N: 11.63%

Found: C: 69.81%; H: 5.68%; N: 11.51%

The material gives a positive Ehrlich test and exhibits infrared absorptions which are typical for the N-H bond of indole (3450, 3320, 3000 cm^{-1}) and a carbonyl absorption (1692 cm^{-1}) but no amide N-H deformation band (ca. 1600 cm^{-1}). Its ultraviolet spectrum has a $\lambda_{max.}$ at 281 mμ, which is typical for indole-3-carbonyl derivatives. It is insoluble in $NaHCO_3$ and in 5% NaOH. This product was not investigated further.

2-Chloro-6-nitrophenylpyruvic Acid: Diethyl oxalate (67.5 ml., 72.7 g., 0.50 mole) (dried over $CaCl_2$ and distilled 181-182°) was added dropwise to a chilled, well stirred solution of sodium ethoxide, made by dissolving freshly cut sodium metal (11.5 g., 0.50 mole) in 190 ml. of absolute ethanol. 2-Chloro-6-nitrotoluene (86 g., 0.50 mole) (practical grade, recrystallized from aqueous methanol) was gradually added to the reaction mixture. The blood red mixture that resulted was stirred at room temperature for 45 minutes and at reflux temperature for 45 minutes. The mixture was poured into 250 ml. of water and steam distilled until the distillate was no longer turbid (distillation time: 1.5 hours; distillate: 2 ℓ). The hot, residual solution was filtered to remove tarry impurities and acidified with HCl. A red, oily precipitate formed and crystallized to a brown solid, with pale yellow crystals forming in the supernate. The crude material (61 g.) contained large amounts of an ether insoluble, high melting impurity, which was removed by dissolving the crude material in ether, filtration and evaporation to a red oil which crystallized to a brown solid (56.5 g., 0.23 mole, 46% yield) when scratched.

A sample, recrystallized twice from hexane, gave yellow crystals, m.p. 110-113° (Lit. 114-115°) (30).

4-Chloroindole-2-carboxylic Acid: Crude 2-chloro-6-nitrophenylpyruvic acid (47.5 g., 0.195 mole) was dissolved in 500 ml. of warm 5N NH_4OH and the solution added to a warm ammoniacal suspension of ferrous hydroxide, freshly prepared by addition of 135 ml. of 15N NH_4OH to a warm solution of $FeSO_4 \cdot 7H_2O$ (327 g., 1.18 mole) in 1125 ml. of water. The mixture was boiled for 5 minutes to coagulate the precipitate, filtered, while hot, over Celite and the precipitate washed with six 75 ml. portions of warm 5N NH_4OH. The filtrate was acidified with HCl and buff colored crystals (30.0 g., 0.154 mole, 79% yield) precipitated.

The crude product was freed from sulfate impurities in the following manner: crude 4-chloroindole-2-carboxylic acid (35.0 g.) was suspended in 750 ml. of 0.7 N HCl containing a trace of $BaCl_2$ and the mixture extracted with four 125 ml. portions of ether. The combined ether extracts were washed four times with water containing a trace of $BaCl_2$, the last washing showing no turbidity. The ether solution was dried over $CaCl_2$ and evaporated to yield tan needles (21.5 g.), m.p. 249-252° (decomp.) (Lit. 259-260°) (30).

4-Cyanoindole: Sulfate free 4-chloroindole-2-carboxylic acid (21.2 g., 0.108 mole) was mixed with cuprous cyanide (15.8 g., 0.085 mole) and 80 ml. of quinoline, and the mixture maintained at reflux temperature for 20 hours. The hot mixture was poured into 1 ℓ. of a mixture of 80 ml. of 12N HCl and crushed ice. After all of the ice had

melted, the mixture was filtered and the filtrate extracted with five 75 ml. portions of ether. The combined extract, and an additional 100 ml. of ether, were used to continuously extract the tarry precipitate for 24 hours. The ether extract was washed with five 100 ml. portions of 1N HCl and three 100 ml. portions of water, dried over Na_2SO_4 and evaporated to yield crude product (9.4 g., 0.064 mole), melting over a wide range near 105° C. Recrystallization from water, with treatment by Norite, gave long, colorless needles (4.85 g., 0.033 mole), m.p. 119-120° (Lit. 120-121°) (30). Additional product (0.60 g., 0.004 mole, 35% total yield) was obtained by washing the filtered Norite with boiling water.

Indole-4-carboxylic Acid: 4-Cyanoindole (2.5 g., 0.0171 mole) was suspended in 25 ml. of 20% KOH solution and the mixture maintained, with stirring, under a nitrogen atmosphere, at reflux temperature for 20 hours. The mixture was cooled, diluted with 50 ml. of water and extracted twice with ether. The aqueous phase was boiled with Norite and filtered. The filtrate, which was still colored, was saturated with nitrogen and acidified with HCl. The flocculent buff colored precipitate (2.60 g., 0.0160 mole), m.p. 212-213° (decomp.) was collected and dried under nitrogen. The product (2.20 g.) was decolorized with Norite and recrystallized from water to obtain colorless needles (1.75 g., 85% yield), m.p. 211.5-214.0° (decomp.) (Lit. 213-214°) (30).

Analysis:	Calculated for $C_9H_7NO_2$(161):	C: 67.07%; H: 4.38%; N: 8.69%
	Found:	C: 67.04%; H: 4.35%; N: 8.66%
		C: 66.93%; H: 4.41%; N: 8.74%

Indole-4-carboxamide: 4-Cyanoindole (2.9 g., 0.020 mole) was added to a mixture of IRA-400 (OH form) anion exchange resin (10 g.) in 500 ml. of carbonate free water and the mixture maintained at reflux temperature for 18 hours. The mixture was filtered, while hot, and the filtrate evaporated, *in vacuo*, to 30 ml. An oily precipitate appeared and crystallized immediately on addition of a seed crystal to form a white solid (2.05 g., 0.013 mole). Additional crude product (0.20 g., 0.001 mole) was obtained by evaporation of the supernate. Crude product (1.8 g.) was recrystallized from chloroform, decolorizing with Norite, to obtain colorless needles (1.1 g., 43% yield), m.p. 141-142° .

Analysis: Calculated for $C_9H_8N_2O$:(160): C: 67.48%; H: 5.03%; N: 17.49%
Found: C: 67.42%; H: 4.92%; N: 17.63%
C: 67.50%; H: 5.01%: N: 17.54%

p-Chlorophenylhydrazine Hydrochloride: A solution of $NaNO_2$ (11.7 g., 0.17 mole) in 25 ml. of water was gradually added to a cold suspension of p-chloroanilinium chloride in HCl, made by addition of p-chloroaniline (18.6 g., 0.123 mole) to 900 ml. of cold 6N HCl. The temperature of the reaction mixture was maintained below 5°, during addition of the nitrite, by addition of ice. A solution of $SnCl_2 \cdot H_2O$ (65.9 g., 0.29 mole) in 60 ml. of 12 N HCl was added immediately, and with stirring, to the diazotized solution. A voluminous, white precipitate appeared almost immediately and was collected by suction filtration and air dried. The crude material was dissolved in 1 *l*. of water and the solution saturated with H_2S. The mixture was filtered to remove the

yellow precipitate that formed and the filtrate boiled with Norite and re-filtered. The colorless filtrate slowly developed an orange color. When the solution was evaporated, in vacuo, to ca. 200 ml., large amounts of precipitate appeared and, after further evaporation of the solvent, product (16.8 g., 0.083 mole, 68% yield), m.p. 210-222° (decomp.), darkens to an orange color, ca. 185° (Lit. 225-230°) (38), was obtained.

Pyruvic Acid p-Chlorophenylhydrazone: A solution of pyruvic acid (6.5 ml., 8.25 g., 0.095 mole) in 100 ml. of water was added to a solution of p-chlorophenylhydrazine hydrochloride (16.8 g., 0.083 mole) in 350 ml. of water. A yellow precipitate (10.7 g., 0.050 mole, 60% yield), m.p. 185-189°, (Lit. 199°) (39) was collected.

Ethyl Pyruvate p-Chlorophenylhydrazone: I. (From pyruvic acid p-chlorophenylhydrazone): A chilled suspension of pyruvic acid p-chlorophenylhydrazone (10.7 g., 0.050 mole) in 250 ml. of anhydrous ethanol was saturated with anhydrous HCl. The resultant solution was boiled to expel excess HCl and mixed with 300 ml. of water. When the solution was cooled, yellow product (7.9 g., 0.0352 mole), m.p. 120-125° crystallized. Additional product (1.5 g., 0.0065 mole, 83% total yield), m.p. 139-140° (Lit. 138°) (39), was obtained by reduction of the volume of the mother liquor.

II. (By the Japp-Klingemann method (40)): To a solution of ethyl α-methylacetoacetate (50 g., 0.348 mole) in 250 ml. of 95% ethanol, at 0°, were added 50% aqueous KOH (130 g., 1.16 mole), crushed ice (ca. 200 g.), and a solution of p-chlorophenyldiazonium chloride, freshly

prepared from p-chloroaniline (45 g., 0.353 mole) in 160 ml. of 10 N HCl and $NaNO_2$ (45 g., 0.65 mole). A brick red oil immediately precipitated and slowly crystallized. The mixture was allowed to stand overnight and filtered, with suction. Considerable amounts of oil were drawn through the filter, leaving a crude solid behind. Recrystallization of the solid gave orange platelets (31 g., 0.124 mole), m.p. 129-136°. The filtrate was extracted with ether, the ether dried over Na_2SO_4 and evaporated to dryness. The residue was recrystallized from aqueous ethanol to give additional crude product (35.5 g., 0.148 mole, 80% total yield).

Ethyl 5-Chloroindole-2-carboxylate: Ethyl pyruvate p-chlorophenylhydrazone (66.5 g., 0.276 mole) was mixed with polyphosphoric acid (135 g.) and the mixture heated with stirring. At ca. 60°, an exothermic reaction occurred, the reaction temperature rising to 130°. The temperature was maintained at 140-145° for 15 minutes and then cooled to 70° . An ice-water mixture (250 cc.) was added and the solid that formed was collected by filtration. This solid was dissolved in ether, the ethereal solution dried over Na_2SO_4 and evaporated to a yellow-brown residue. Recrystallization from methanol gave yellow needles (27.0 g., 0.121 mole, 44% yield), m.p. 164-168° (Lit. 167-168°) (27).

5-Chloroindole-2-carboxylic Acid: Ethyl 5-chloroindole-2-carboxylate (27.0 g., 0.121 mole) was dissolved in a solution of KOH (122 g., 2.2 mole) in 1250 ml. of 90% denatured ethanol and the solution maintained at reflux temperature for 2.5 hours. The reaction mixture

was cooled and poured into 1 ℓ. of 2.5 N HCl. Yellow crystals (23.5 g., 0.120 mole, 99% yield), m.p. 259-266° (decomp.) (Lit. 289-290°) (27), with some sublimation at lower temperature, crystallized, and were collected and dried in vacuo.

5-Cyanoindole: 5-Chloroindole-2-carboxylic acid (24.5 g., 0.125 mole), cuprous cyanide (18.3 g., 0.098 mole), and 95 ml. of quinoline were mixed and the mixture maintained at reflux temperature for 24 hours. The hot reaction mixture was poured into 1 ℓ. of a mixture of 95 ml. of 12N HCl and ice. After all of the ice had melted, the dark green precipitate that formed was filtered and the filtrate washed with four 75 ml. portions of ether. The combined ethereal extracts and an additional 100 ml. of ether were used to continuously extract the precipitate for 24 hours. The ethereal extract was washed with five 100 ml. portions of 1N HCl and three 100 ml. portions of water, dried over Na_2SO_4, and evaporated to an oil (7.3 g., 0.0514 mole) which slowly crystallized. Recrystallization from aqueous ethanol gave crude brown solid (4.6 g., 0.0324 mole, 26% yield). A sample was recrystallized from water, decolorizing with Norite, to obtain colorless platelets, m.p. 106.5-107.0° (Lit. 104-106°) (41).

Indole-5-carboxylic Acid: 5-Cyanoindole (2.3 g., 0.0162 mole) was suspended in 25 ml. of 20% KOH and the mixture maintained at reflux temperature for 20 hours. The mixture was cooled, diluted with 50 ml. of water and extracted with ether. The aqueous phase was boiled with Norite, filtered and acidified with HCl. Orange crystals (2.2 g., 0.0137 mole, 84% yield) precipitated and were collected under a nitrogen

atmosphere. The product was decolorized by boiling a benzene solution of the material with Norite, filtering and adjusting the warm filtrate to its cloud point by addition of hexane. When the solution cooled, a colorless powder, m.p. 209.5-211.0° (decomp.) (Lit. 208-209°) (31) was obtained.

Analysis: Calculated for $C_9H_7NO_2$(161): C: 67.07%; H: 4.38%; N: 8.69%

Found: C: 67.19%: H: 4.48%; N: 8.92%

Indole-5-carboxamide: 5-Cyanoindole (1.18 g., 0.00825 mole) was suspended in 400 ml. of boiling, carbonate free water and IRA-400 (OH form) anion exchange resin (3.0 g.) was added to the reaction mixture. The mixture was maintained at reflux temperature for 16 hours, filtered, while hot, and the filtrate evaporated to dryness. The white residue was recrystallized from water to yield shiny, colorless needles (0.52 g., 0.00325 mole), m.p. 165.5-167.0°. Reduction of the volume of the supernate gave additional product (0.10 g., 0.00063 mole, 47% total yield).

Analysis: Calculated for $C_9H_8N_2O$(160): C: 67.48%; H: 5.03%; N: 17.49%

Found: C: 67.18%; H: 4.87%; N: 17.43%

4-Chloro-2-nitrophenylpyruvic Acid: Diethyl oxalate (67.5 ml., 72.7 g., 0.50 mole) was added dropwise to a sodium ethoxide solution prepared by addition of freshly cut sodium (11.5 g., 0.50 mole) to 200 ml. of absolute ethanol. 4-Chloro-2-nitrotoluene (86 g., 0.50 mole) was added, in portions, and the blood red reaction mixture maintained at reflux temperature for 40 minutes. The reaction mixture was poured

into 250 ml. of water and steam distilled until the distillate was no longer turbid. The warm, residual solution was filtered to remove tarry impurities and acidified with HCl. A copious, red, oily precipitate formed, which crystallized on cooling. The crystals were collected by suction filtration, washed with dilute HCl, and dried, in vacuo, to obtain crude product (63.5 g., 0.261 mole, 52% yield). A sample was recrystallized from benzene to obtain yellow crystals, m.p. 138-139° (Lit. 136°) (27). Although, as in the case of 2-chloro-6-nitrophenylpyruvic acid, the crude product contained an ether insoluble impurity, no attempt was made to remove this impurity and the crude material was found to be adequate for the subsequent reaction.

6-Chloroindole-2-carboxylic Acid: Crude 4-chloro-2-nitrophenylpyruvic acid (63.5 g., 0.26 mole) was dissolved in 500 ml. of warm 5N NH_4OH and the solution added to a warm, ammoniacal ferrous hydroxide suspension, freshly prepared by addition of 180 ml. of 15N NH_4OH to a warm solution of $FeSO_4 \cdot 7H_2O$ (445 g., 1.60 mole) in 1.5 ℓ. of water. The mixture was boiled to coagulate the precipitate and filtered through Celite and extremely retentive filter paper, washing the precipitate with seven 75 ml. portions of warm 5N NH_4OH. The filtrate was refiltered through coarse paper to remove a small amount of violet precipitate and acidified with HCl to obtain crude, colorless product (22 g., 0.113 mole). The material was suspended in 500 ml. of 0.8N HCl, containing a trace of $BaCl_2$ and extracted four times with ether. The combined ether extract was washed with water, containing a trace of $BaCl_2$, until the

aqueous washing was no longer turbid, dried over $CaCl_2$, and evaporated to tan residue (16.0 g.), m.p. 231-235° (Lit. 242°) (27) (decomp.), with sublimation above 200°.

6-Cyanoindole: Sulfate free 6-chloroindole-2-carboxylic acid (16.0 g., 0.082 mole), cuprous cyanide (12.0 g., 0.064 mole), and 65 ml. of quinoline were mixed and the mixture maintained at reflux temperature for 20 hours. The hot reaction mixture was poured into 1 ℓ. of a mixture of 65 ml. of 12N HCl and crushed ice. After all of the ice had melted, the mixture was filtered and the filtrate extracted five times with ether. The combined ethereal extract was used to continuously extract the precipitate for 24 hours. The ethereal extract was washed five times with 100 ml. of 1N HCl and three times with water, dried over Na_2SO_4, and evaporated to a brown solid (6.1 g., 0.0427 mole, 52% yield). A sample was recrystallized from water to obtain long, colorless needles, m.p. 128.5-130.0° (Lit. 129-130°) (42).

Indole-6-carboxylic Acid: Crude 6-cyanoindole (3.0 g., 0.021 mole) was suspended in 30 ml. of 20% KOH and the mixture stirred and maintained at reflux temperature for 24 hours. The mixture was diluted with 150 ml. of water and washed three times with ether. The aqueous phase was boiled with Norite, filtered, and acidified with HCl to obtain tan crystals (1.85 g., 0.0115 mole). Recrystallization from water and decolorizing with Norite gave pale yellow needles (1.10 g., 0.0068 mole, 33% yield). Further attempts at decolorizing, including boiling a solution of product (0.5 g.) in 250 ml. of water with Norite A (0.5 g.), under a

nitrogen atmosphere, for 24 hours, had no effect in removing the yellow color. The product, m.p. 250-252° (decomp.) (Lit. 243-244°) (42) dissolved in equivalent amounts of base to give solutions with no perceptible color and whose inhibitory properties toward α-chymotrypsin were independent of the age of the solution.

Analysis: Calculated for $C_9H_7NO_2$(161): C: 67.07%; H: 4.38%; N: 8.69%

Found: C: 66.84%; H: 4.49%; N: 8.53%

Indole-6-carboxamide: Crude 6-cyanoindole (0.75 g., 0.00525 mole) was suspended in 250 ml. of hot, carbonate free water and IRA-400 (OH form) anion exchange resin (2.6 g.) added to the suspension. The mixture was maintained at reflux temperature for 12 hours, filtered, and the filtrate evaporated, in vacuo, to ca. 75 ml. and cooled. Light tan platelets (0.34 g., 0.00212 mole, 40% yield), m.p. 188.5-190.5°, crystallized. Recrystallization from water and decolorizing with Norite gave colorless needles, m.p. 188.5-190.5°.

Analysis: Calculated for $C_9H_8N_2O$ (160): C: 67.48%; H: 5.03%; N: 17.49%

Found: C: 67.66%; H: 5.10%; N: 17.55%

o-Chlorophenylhydrazine Hydrochloride: o-Chloroaniline (45 g., 0.327 mole) was dissolved in 3 ℓ. of 2N HCl and the solution cooled to 4°. $NaNO_2$ (27 g., 0.392 mole) was added in portions to the cold solution. A solution of $SnCl_2 \cdot 2H_2O$ (152 g., 0.392 mole) in 125 ml. of 12N HCl was added immediately to the diazotized solution. The solution turned orange and a small amount of pink crystals appeared. The mixture was concentrated, in vacuo, to ca. 750 ml. and cooled. A large

amount of pink needles precipitated. The product was not dried but used directly in the synthesis of pyruvic acid o-chlorophenylhydrazone.

Pyruvic Acid o-Chlorophenylhydrazone: The moist, crude precipitate, described in the previous paragraph, was dissolved in 1350 ml. of warm water and the solution clarified by filtration. A solution of pyruvic acid (32 g., 0.364 mole) in 275 ml. of water, was added, with stirring. A large amount of pale yellow precipitate formed and was collected. Recrystallization from ethanol gave yellow crystals (36.7 g., 0.174 mole), m.p. 165-172° (Lit. 178°) (43) and softening ca. 150°.

Ethyl Pyruvate o-Chlorophenylhydrazone: I. (From pyruvic acid o-chlorophenylhydrazone): Pyruvic acid o-chlorophenylhydrazone (34.7 g., 0.164 mole) was suspended in 1 ℓ. of absolute ethanol and the mixture cooled to 0° and saturated with anhydrous HCl, after which all of the solid had dissolved. The solution was evaporated, in vacuo, until a precipitate formed. The precipitate redissolved when the mixture was warmed on the steam bath and the solution was adjusted to its cloud point by addition of water and, when the solution was cooled, long, pale yellow needles (16.1 g., 0.067 mole), m.p. 64-67° (Lit. 71°) (43) crystallized. Additional product (17.8 g., 0.074 mole, 86% total yield) was obtained by reduction of the volume of the mother liquor.

II. (By the Japp Klingemann method): To a solution of ethyl α-methylacetoacetate (25 g., 0.174 mole) in 125 ml. of 95% ethanol, cooled to 0°, were added cold, 50% KOH solution (65 g., 0.58 mole), crushed ice (100 g.), and a solution of o-chlorophenyldiazonium chloride, freshly

prepared by addition of $NaNO_2$ (22.5 g., 0.326 mole), in portions, to a solution of o-chloroaniline (22.5 g., 0.176 mole) in 550 ml. of 1.5 N HCl, which had been cooled to 5°. A brick red oil precipitated immediately. The mixture was extracted five times with ether and the ether dried over Na_2SO_4 and evaporated to a red oil. A portion of the oil was recrystallized from aqueous methanol to obtain orange platelets, m.p. 60-64°. These crystals were used to seed the recrystallization of the main body of product and a red-orange solid (29.5 g., 0.123 mole, 71% yield) was obtained in this way.

Ethyl 7-Chloroindole-2-carboxylate: Ethyl pyruvate o-chlorophenylhydrazone (40 g., 0.166 mole) was mixed with polyphosphoric acid (40 g.) and the mixture heated, with stirring, to 230°, whereupon a vigorous reaction, with extensive foaming, occurred. The mixture was cooled to 60° and 400 ml. of water was added. The black solid that formed was collected by filtration, air dried, and continuously extracted with 500 ml. of ether for 15 hours. The red ethereal extract was dried over Na_2SO_4 and evaporated, in vacuo, to a yellow brown solid which was shown by infrared analysis to be a 3:1 mixture of ethyl 7-chloroindole-2-carboxylate and starting material. This mixture was recrystallized from 700 ml. of hexane, with a considerable amount of black tar remaining undissolved in the boiling solvent. On cooling the solution, yellow crystals (5.1 g., 0.0229 mole), m.p. 98-105°, were obtained. Reduction of the volume of the mother liquor to ca. 250 ml. gave material (8.4 g.), m.p. 65-105°, and recrystallization of this material from 250 ml. of hexane

gave product (4.6 g., 0.0206 mole), m.p. 98-106°. On reduction of the mother liquor of the second recrystallization to ca. 125 ml., product (0.65 g., 0.0029 mole, 28% total yield), m.p. 102-105° (Lit. 105°) (27), was obtained. The residues of both mother liquors (18.5 g.) were too rich in starting material for convenient separation of product. These mixtures were saved for subsequent reaction with polyphosphoric acid.

7-Chloroindole-2-carboxylic Acid: Ethyl 7-chloroindole-2-carboxylate (14.1 g., 0.0632 mole) was dissolved in a solution of KOH (62 g.) in 750 ml. of 90% ethanol and the solution maintained at reflux temperature for 2.5 hours The solution was cooled and poured, with stirring, into 140 ml. of 1N HCl. Brown powder (7.8 g., 0.0400 mole), m.p. 230-235° (decomp.) (Lit. 234-236°) (27), crystallized. Evaporation of the mother liquor to remove most of the ethanol gave additional product (3.6 g., 0.0185 mole, 93% total yield).

7-Cyanoindole: 7-Chloroindole-2-carboxylic acid (12.1 g., 0.062 mole), cuprous cyanide (9.1 g., 0.049 mole), and 45 ml. of quinoline were mixed and the mixture maintained at reflux temperature for 24 hours. The hot reaction mixture was poured into 600 ml. of a mixture of 45 ml. of 12 N HCl and crushed ice. After all of the ice had melted, the mixture was filtered and the filtrate extracted five times with 75 ml. portions of ether. The combined extract was used to continuously extract the precipitate for 24 hours. The extract was dried over Na_2SO_4 and evaporated, in vacuo, to a red oil which partially crystallized in yellow needles. The oil crystallized as a crude, dark solid (1.7 g., 0.0119 mole,

19% yield) from aqueous ethanol. A sample was recrystallized from aqueous acetone to give needles, m.p. 101-102.5° (Lit. 96°) (44).

Indole-7-carboxylic Acid: Crude 7-cyanoindole (1.0 g., 0.0070 mole) was suspended in 10 ml. of 20% KOH and the mixture maintained at reflux temperature for 20 hours. The reaction mixture was cooled, diluted with 80 ml. of water, and extracted three times with ether. The aqueous phase was boiled with Norite, filtered, and the filtrate acidified with HCl. Pale yellow material (0.65 g., 0.0041 mole) precipitated. The material was recrystallized from water, decolorizing with Norite, to obtain long, colorless needles (0.38 g., 0.00256 mole, 37% yield), m.p. 206-212° (Lit. 198-199°) (44).

Analysis: Calculated for $C_9H_7NO_2$(161): C: 67.07%; H: 4.38%; N: 8.69%

Found: C: 67.22%; H: 4.49%; N: 8.57%

Indole-7-carboxamide: Crude 7-cyanoindole (0.6 g., 0.0042 mole) was suspended in 250 ml. of carbonate free water, containing IRA-400 (OH form) anion exchange resin (2.5 g.), and the mixture maintained at reflux temperature for 10 hours. The reaction mixture was filtered, while hot, and, when the filtrate was cooled, white platelets (0.24 g., 0.0015 mole) crystallized. Evaporation of the mother liquor, in vacuo, and recrystallization of the residue from water, gave additional product (0.11 g., 0.0007 mole, 52% yield). Recrystallization from water, decolorizing with Norite, gave colorless laminae, m.p. 205-207°.

Analysis: Calculated for $C_9H_8N_2O$(160): C: 67.48%; H: 5.03%; N: 17.49%

Found: C: 67.62%; H: 5.15%; N: 17.67%

L-Tyrosine Methyl Ester Hydrochloride: L-Tyrosine (50.0 g., 0.276 mole) was suspended in 300 ml. of reagent grade methanol, the mixture cooled to 4°, and saturated with anhydrous HCl. About half the tyrosine dissolved during saturation. The mixture was maintained at reflux temperature overnight, all of the remaining tyrosine dissolving. The solution was evaporated, in vacuo, to obtain a white, pasty mass of crude L-tyrosine methyl ester hydrochloride.

N-Acetyl-L-tyrosine Methyl Ester: The crude L-tyrosine methyl ester hydrochloride was dissolved in 50 ml. of warm water and the solution cooled in a salt-ice bath. $NaHCO_3$ (47 g., 0.56 mole) was added, in portions, with stirring. During the first part of the addition of bicarbonate, CO_2 was formed, and a white solid precipitated. One *l.* of ethyl acetate was added to the mixture and the biphasic system stirred vigorously while acetic anhydride (21.5 ml., 22.4 g., 0.219 mole) was added dropwise, over a period of 10 minutes. Stirring was continued for another hour and, at the end of this time, large amounts of a white, crystalline precipitate had formed. The material was collected by filtration and washed well with water, m.p. 113-116°. Recrystallization from water gave product (47.4 g., 0.200 mole, 73% yield from L-tyrosine), m.p. 136.5-138.0° (Lit. 136-137°) (45), $[\alpha]_D$ + 24.2 ± 0.2° (Lit. 29.7°) (45).

Sodium N-Acetyl-L-tyrosinhydroxamate: A solution of methanolic hydroxylamine was prepared by addition of a sodium methoxide solution, prepared by addition of freshly cut sodium (5.25 g., 0.228 mole) to 80 ml. of reagent grade cold methanol, to a solution of hydroxylamine hydrochloride (12.2 g., 0.176 mole) in 70 ml. of warm (42°) methanol. The mixture was stirred for 45 minutes, cooled in a salt-ice bath, and filtered to remove the NaCl. N-Acetyl-L-tyrosine methyl ester (10.4 g., 0.0439 mole) was dissolved in the hydroxylamine solution and the solution stirred for 15 minutes, after which large amounts of white crystalline material precipitated. The solution was cooled overnight and the precipitate collected and dried, in vacuo, to obtain product (8.85 g., 0.0340 mole, 70% yield), m.p. 190-190.5°, darkening above 180° (Lit. 190.5-191.0°) (19), $[\alpha]_D$ = + 34.8° ± 0.1° (5% solution in an equivalent amount of HCl) (Lit. + 35°) (19).

Analysis: Calculated for $C_{11}H_{13}N_2O_4Na$(260.2):

C: 50.77%; H: 5.03%; N: 10.77%

Found: C: 50.88%; H: 5.00%; N: 10.73%

α-Chymotrypsin: Armour bovine α-chymotrypsin, lot #283, was used for all kinetic experiments.

Analysis: N: 15.09, 15.19% protein nitrogen (Dumas method)

Apparatus:

All kinetic experiments were performed by measuring the rate of production of acid by means of a pH-stat. The apparatus (46) and operating procedure (47) have been described in detail. The operating

principle of this apparatus is the detection, by glass electrodes, of drops in pH below a specified value with consequent activation of a microsyringe, which then adds sufficient standard base to maintain the desired pH, the amount of base added being recorded as a function of time on chart paper.

Kinetic Procedure:

Into a 20 ml. beaker was pipetted: from 1 to 5 ml. of a freshly made stock solution of sodium N-acetyl-L-tyrosinhydroxamate in an equivalent amount of HCl, a volume of NaCl solution, whose concentration was equal to that of the substrate stock solution, such that the total volume of substrate stock and dilute salt solutions was constant for all runs in a particular experiment, 1.0 ml. of a concentrated NaCl solution, such that a total of 2.0 millimoles of NaCl were added to the solution, and sufficient inhibitor stock solution, or water, when necessary, to bring the volume of solution up to 9.0 ml., neglecting volume changes on mixing. The beaker was placed in a specially fixed water jacket, thermostated at 25.0° C, and the entire assembly raised, by means of an air jack, so that the glass electrodes, stirrer, and the tip of the standard base syringe were immersed in the solution. Care was taken to make sure that none of these components was in contact with the beaker, or each other. The reaction cell assembly had a Lucite cover and nitrogen was constantly flowing above the solution. NaOH (1 N) was carefully added, from a hand syringe, until the pH was within 0.03 units of the desired pH, 7.60. The titrimeter was set for constant pH titration at 7.60 and the drive speed, which controls the increment of

standard base added for a particular deviation from the desired pH value, set at its minimum. The titrimeter was activated and, in this way, an accurate adjustment of pH was made. Approximately 2.5 ml. of a freshly made enzyme stock solution was transferred to a 10 ml. beaker, care being taken to avoid undue foaming, and a separate set of glass electrodes and stirrer immersed in the solution. This assembly also was covered by a Lucite disk and nitrogen was passed over the solution. The solution was neutralized, by careful addition of 1N NaOH from a hand syringe, to pH 7.6 $\pm$ 0.2. A syringe, in a double stop holder, weight calibrated to deliver 1.00 ml., was immediately rinsed with a small amount of the neutralized enzyme solution and then filled. The titrimeter was set for maximum drive speed and 1.00 ml. of the enzyme stock added to the reaction solution. Due to mild buffering action of the reaction systems, a time lag of about 10 seconds before the first addition of base was observed. This interval was used to transfer another 1.5-2.5 ml. of enzyme stock solution to the 10 ml. beaker in preparation for the next velocity determination. In this way, the pH of the unused, neutralized enzyme solution was decreased below pH 6, to a range in which the enzyme was stable. As soon as it became apparent that the drive speed was set too high, as indicated by large discontinuities in the chart trace, the drive speed was lowered until the discontinuities were minimized, without allowing the rate of addition of base to fall below that necessary to maintain the desired pH.

Considerable difficulty was encountered, at first, in this adjustment. Because of the buffering of the system, it was not immediately apparent when the drive speed was insufficient and the titrimeter would "fall behind" the reaction to a considerable extent before a decrease in pH could be observed visually on the meter. Since the increment of base added, at any drive speed, is proportional to the deviation from the "poise" pH, this would often cause upward curvature of the kinetic trace as the rate of addition of base was belatedly increased. When it became apparent, by the accumulation of a large amount of data, that the kinetics for this system were linear over an eight minute interval, it was decided to run at slightly higher drive speeds, since the frequent occurrence of small discontinuities in the trace represent a random uncertainty rather than a systematic error. Two titrimeters, both manufactured by International Instrument Company, were used in this work. The only significant difference in their operation was that one had a continuous and the other an incremental drive speed control. This had no significant effect on the results and determinations made with one titrimeter were shown, by statistical analysis, to be identical with determinations for the same reaction system made with the other. As experience with the operation of the apparatus was gained, final adjustment of the drive speed could be made during the first minute of the run. Furthermore, as soon as sufficient data to allow crude estimation of the expected velocity for a particular system was obtained, the drive speed could be preset. The reactions were followed for at least eight minutes and the

remainder of this time was used to prepare a new reaction solution for the next run.

Substrate and inhibitor stock solutions were made up by dissolving accurately weighed, analytically pure samples, in equivalent amounts of acid or base, when necessary, and diluting to the mark of the appropriate volumetric flasks with carbonate free water. Enzyme solutions were made by dissolving the accurately weighed, crystalline enzyme in accurately measured volumes of carbonate free water, care being taken to avoid the formation of foams during mixing. With the exception of NaCl solutions, all solutions were made on the same day as the performance of the experiment. $(S)_o$ was varied from $5 \cdot 10^{-3}$ M to $7 \cdot 10^{-2}$ M. $(E)_o$ was within the limits 0.020 to 0.022 mg. P. N./ml. After a preliminary experiment, K_I was determined, for each inhibitor, in the range $K_I < (I)_o < 3K_I$, if possible. With the exception of indole-2-carboxamide and indole-7-carboxamide no solubility difficulties were encountered. A supersaturated $5 \cdot 10^{-3}$ M indole-2-carboxamide solution was prepared by rapidly cooling a warm solution to 20°C in a water bath and pipetting the desired aliquots into the reaction solutions before crystallization occurred. For convenience, all reaction solutions, containing this inhibitor, were made up at the beginning of the experiment. No apparent change of behavior of the solutions, with time, was observed and reasonably precise results were obtained with this inhibitor. In the case of indole-7-carboxamide, a stock solution of $1.74 \cdot 10^{-3}$ M was prepared. By making up the dilute NaCl and substrate stock solutions in $1.05 \cdot 10^{-3}$ M indole-7-carboxamide solution, an $(I)_o$ of $9.75 \cdot 10^{-4}$M was obtained, without

decreasing the range of $(S)_o$'s.

Calculations: The velocities were determined by a linear, least-squares plot of the chart readings at nine points at one minute intervals. Use of the orthogonal polynomial procedure of Booman and Niemann (48) showed that runs of higher curvature were isolated and random cases and were unrelated to substrate concentration. No significant enzyme or substrate blanks were observed. Since hydroxylamine is 2.5% protonated at pH 7.60, (NaCl) = 0.20 M and 25.0°C (19), the velocities were corrected for buffering by the factor 1.025.

Kinetic constants were calculated by a reiterative Lineweaver-Burk plot (49):

$$(S)_o(E)_o/v_o = K_s/k_3 + (S)_o/k_3$$

where $(S)_o$ and $(E)_o$ are the initial substrate and enzyme concentrations and v_o the velocity at $t = 0$. The reciprocal of the slope, b, is equal to k_3, the rate constant for the decomposition of the enzyme-substrate complex to products, and the ratio of the intercept, a, to the slope equals K_s, the apparent equilibrium constant of the complex. The experimental plot was subject to statistical reiteration, by which the "worst point," j, such that:

$$z_j = |y'_j - y_j| \Big/ \left[(n+1)/n \sum_{i=1}^{n} (x_i - \bar{x})^2 + (x_j - \bar{x})^2 \right]^{1/2}$$

where: $y_j = ((S)_o(E)_o/v_o)_j$ $\quad x_j = (S)_{oj}$

$y'_j = a + bx_j$ $\quad \bar{x}$ = average x

is larger than the z value for all other points, is removed from the data and a new, least squares line plotted. The "worst point" is subjected to a 98% significance "T" test, with respect to the new line and, if it fails, is discarded and a new worst point, with respect to the new line, is chosen and tested. When a worst point passes the significance test, the reiteration is stopped and the current least squares plot used to calculate the kinetic constants.

For systems containing an added inhibitor, I:

$$(S)_o (E)_o/v_o = (K_s/k_3)(1 + (I)/K_I) + (S)_o/k_3$$

where K_I is the dissociation constant of the enzyme-inhibitor complex. A Lineweaver-Burk plot for such a system will be parallel to the plot for the same substrate in the absence of inhibitor and its intercept will be greater by the factor $1 + (I)/K_I$. Therefore:

$$K_I = (I)/((a'/a) - 1)$$

where a' is the intercept of the plot in the presence of inhibitor.

Uncertainties for the slope and intercept values were calculated by standard statistical procedures, assuming that all points of the plot were members of the same, normally distributed family of points. While, due to the reciprocal nature of the plot, this is not strictly true, the method is considered to give a reasonable estimate of the uncertainties.

While it is possible to formally calculate K_I without using the slope of the plot, the uncertainty of the slope cannot be neglected, since

it includes the uncertainty in $(E)_o$, a large potential source of error. However, due to the small magnitudes of the slopes for the systems studied and the increase in the absolute uncertainty of y as the velocities are decreased by added inhibitors, quite large uncertainties in the slope were sometimes encountered, especially in cases for which $(I) > 3K_I$. Therefore, the uncertainty of K_I was estimated by the equation:

$$\sigma_{K_I} = K_I(2\sigma_b/b + \sigma_a/a + \sigma_{a'}/a') \cdot \frac{a'/a}{a'/a-1}$$

Calculations were performed using a "Datatron 220" digital computer. A program was written which would calculate reaction velocities by least squares or orthogonal polynomial procedures and perform a reiterative Lineweaver-Burk plot with these velocities and the corresponding $(S)_o$'s and $(E)_o$'s.

Results:

The data for the α-chymotrypsin catalysed hydrolysis of N-acetyl-L-tyrosinhydroxamide at pH 7.60, 25° and 0.20 M NaCl were found to be in good agreement with the data of Kurtz (19), Jennings (51), and Hogness and Niemann (33).

All inhibitors investigated were found to act in a competitive manner toward the system α-chymotrypsin, N-acetyl-L-tyrosinhydroxamide. The average k_3 for nineteen inhibited runs was $25.5 \pm 2.1 \cdot 10^{-3}$ (m/min.)/(mg. P. N./ml.). While the inhibited runs show a constant k_3, there does seem to be a tendency for k_3 to be lower than in the uninhibited case. This lies, however, just on the border of significance and does not justify the abandonment of the assumption of competitive inhibition. There was no apparent dependence of k_3 on inhibitor concentration.

At least one experiment for each inhibitor was run in the approximate range: $K_I < (I) < 3K_I$. If $(I) < K_I$, a situation encountered in the preliminary runs for several inhibitors, an unprecise K_I value was obtained, due to the inherent uncertainty of measuring small differences in experimental values. If $(I) > 3K_I$, a precise K_I value, based on the ratio of the intercepts, could be obtained but the k_3 value was often highly uncertain since the kinetics of the system were now beginning to approach first order behavior. For all inhibitors except indole-7-carboxamide, the K_I's were determined for at least two different concentrations. In all cases, there was good agreement between the results determined at different inhibitor concentrations. The preferred K_I value

Table 1: α-Chymotrypsin Catalysed Hydrolysis of N-Acetyl-L-tyrosinhydroxamide.

Ref.	$(S)_o$ [a]	(NaCl) M	pH	$(E)_o$ [b]	k_3 [c]	K_s [a]
This thesis	5-46	0.20	7.60	0.021 ±0.001	30.8± 1.0	51.1±2.5
19	5-40	0.04	"	±0.0209	29.4±1.1	53.9±2.2
"	"	0.200	"	"	32.2±1.4	50.4±2.3
"	"	0.500	"	"	42.8±3.0	49.9±3.8
"	"	1.00	"	"	36.6±3.0	33.5±2.7
"	"	2.00	"	"	32.1±0.9	17.8±0.8
50	3-70	0.02 [d]	"	0.04	35.2	47±3
33	5-35	0.30	"	0.209	34	51

a) in units of 10^{-3}M

b) in units of mg. Protein Nitrogen/ml.

c) in units of 10^{-3}M/min./(mg. P. N./ml.)

d) in amine component of THAM

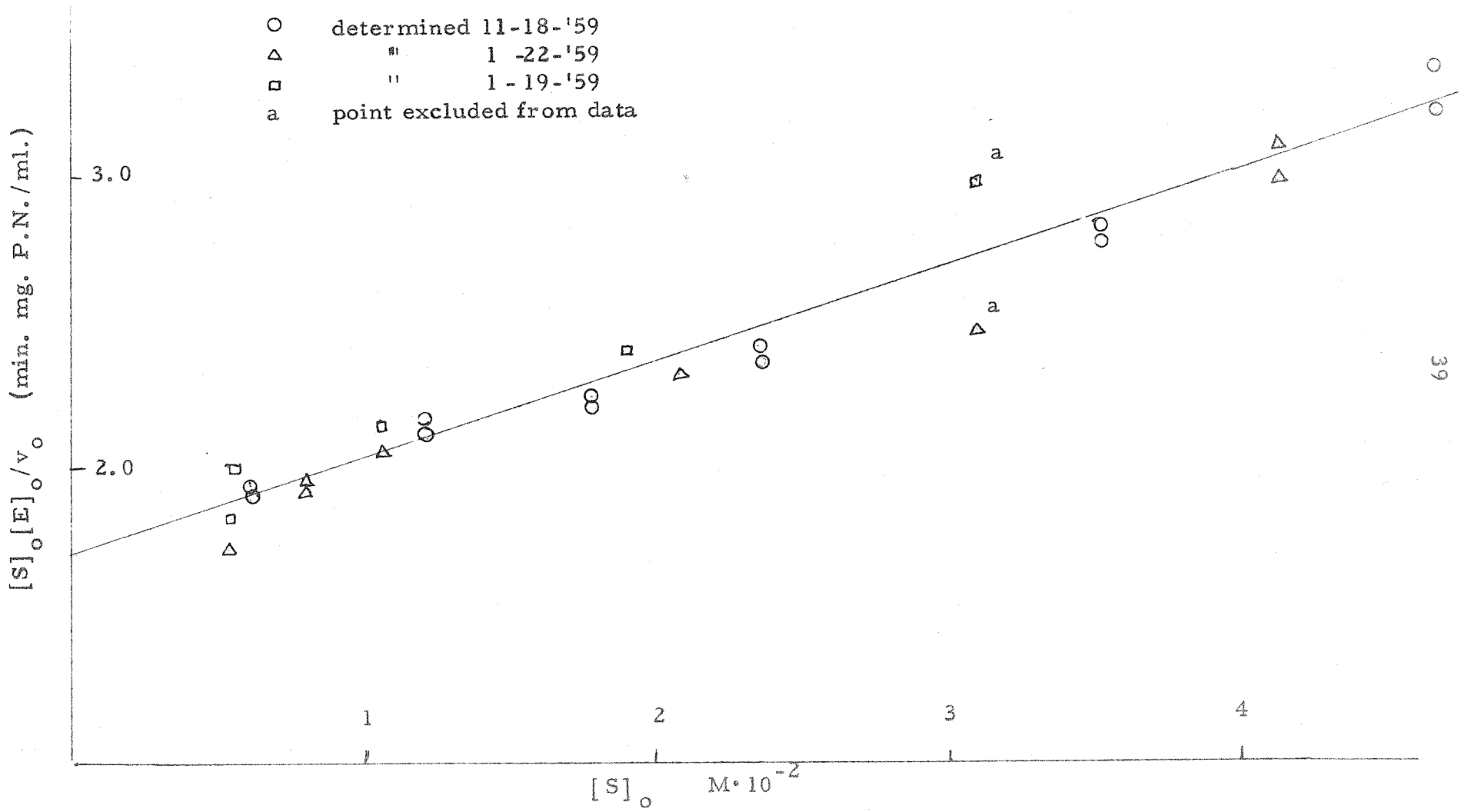


Figure 1. α-Chymotrypsin catalysed hydrolysis of N-acetyl-L-tyrosinhydroxamide at pH 7.60, 25.0° and 0.20 M NaCl (points not corrected for 25% buffering).

is the value, determined in the preferred concentration range, with the smallest uncertainty limits.

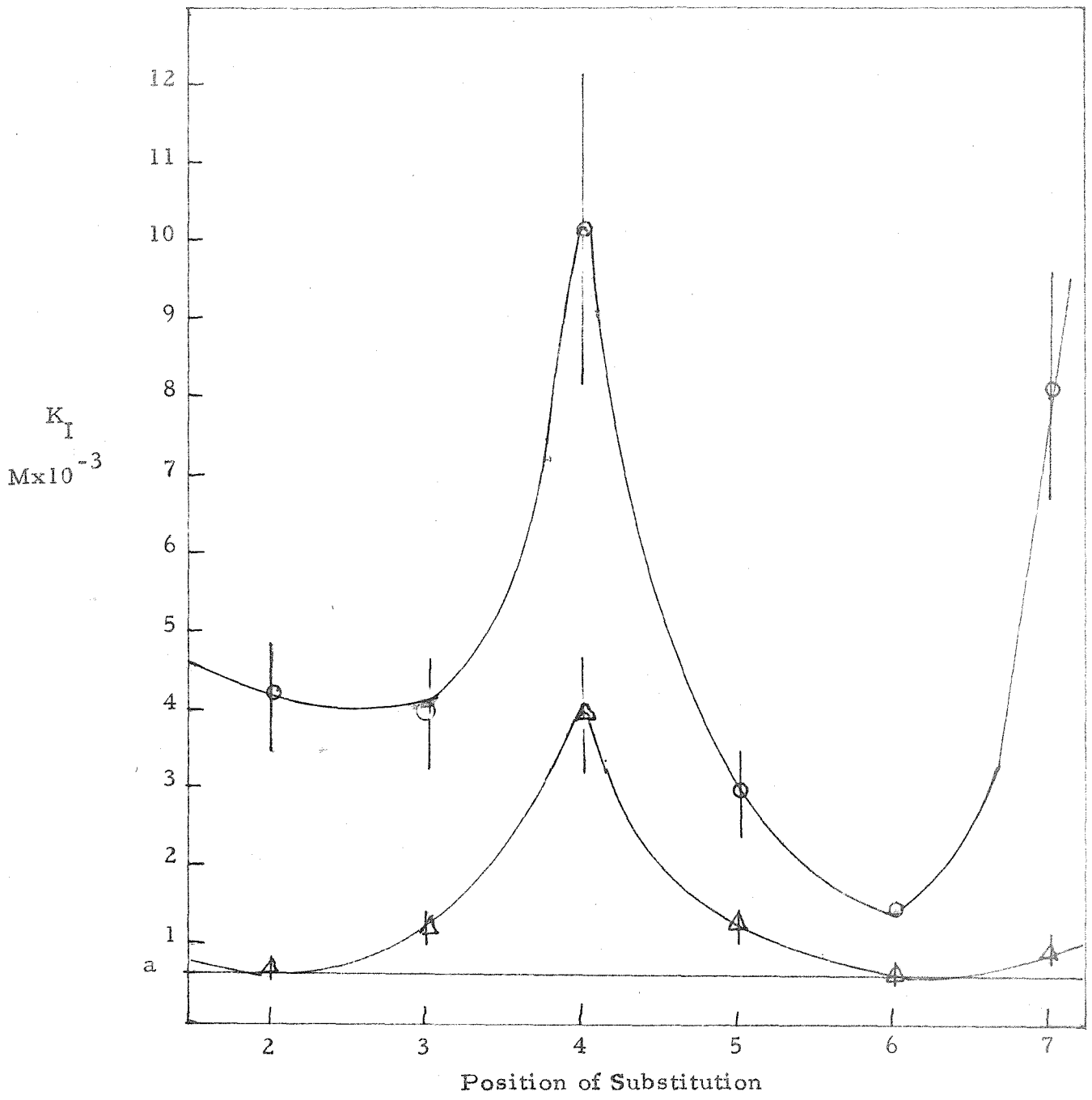
The results of this investigation are shown in table 2 and in figure 2.

Table 2: The Inhibition Constants of Indole, Its Carboxylate Ions and Carboxamides vs. the α-Chymotrypsin Catalysed Hydrolysis of N-Acetyl-L-tyrosinhydroxamide at pH 7.60, 25.0° and 0.20 M NaCl.

Inhibitor	$(I)_o$ [a]	K_I [a]	$\frac{K_{I_{CO_2^-}}}{K_{I_{CONH_2}}}$	$\delta(-\Delta F^o)^{b}_{COO^- \atop CONH_2}$
indole	1.81	0.63±0.10		
"-2-carboxylate ion	7.54	4.2±0.7	6.6	-1.1
"-3- "	5.12	4.0±0.7	3.2	-0.7
"-4- "	13.32	10.2±2.0	2.6	-0.6
"-5- "	4.02	3.0±0.5	2.4	-0.5
"-6- "	4.05	1.48±0.17	2.5	-0.5
"-7- "	10.79	8.2±1.4	8.4	-1.3
			$\frac{K_{I_{CONH_2}}}{K_{I_{indole}}}$	$\delta(-\Delta F^o)_{CONH_2\text{-indole}}$
"-2-carboxamide	1.044	0.64±0.13	1.0	0.0
"-3- "	3.01	1.24±0.20	2.0	-0.4
"-4- "	6.07	4.0±0.7	6.4	-1.1
"-5- "	2.03	1.33±0.22	2.1	-0.4
"-6- "	0.981	0.60±0.09	1.0	0.0
"-7- "	0.975	0.98±0.20	1.6	-0.3

a) in units of 10^{-3} M

b) in units of Kcal/Mole at 25.0° C, to nearest 0.1 Kcal/mole



Inhibition constants of indolecarboxylate ions and indolecarboxamides at pH 7.60, 25.0°, 0.20 M NaCl

○ carboxylate ions

△ amides

a indole $K_I = 0.63 \times 10^{-3}$ M

Discussion:

It can be seen from the data presented that the indolecarboxylate ions are, without exception, weaker inhibitors toward α-chymotrypsin than either indole or their corresponding amides. None of the amides is a more effective inhibitor than indole, a result which implies the absence of multifunctional binding effects in this series of compounds. The observed inhibition constants for both series of inhibitors are highly dependent on the position of substitution, implying that the indole nucleus complexes with the active site of α-chymotrypsin with a preferred orientation.

Both indole-4-carboxylate ion and indole-4-carboxamide are the weakest inhibitors of their respective isomeric series. The fact that the $-\delta(\Delta F^{o})$ between the carboxylate ion and the amide is only 0.6 Kcal/Mole, or close to the minimum observed value, indicates that the nature of the repulsions, acting on these inhibitors, is steric, rather than electrostatic in nature. Thus, in the α-chymotrypsin-indole complex, a neutral group must lie in fairly close proximity to the 4 position of the indole ring.

The complexes of α-chymotrypsin with indole-2- and -7- carboxylate ions both show decreases in stability, relative to their corresponding amides, of greater than 1.0 Kcal/Mole. Since the complex of indole-2-carboxamide is not decreased in stability relative to indole, and indole-7-carboxamide shows a decrease in stability of only 0.3 Kcal/Mole, a

negatively charged group, in the indole-α-chymotrypsin complex, must be situated near the indole nitrogen atom, without being so close as to exert large steric repulsions on the adjacent positions. The negative group may, by its interaction with the indole nitrogen, be a source of added stability for indole complexes and explain the generally low K_I's of indole derivatives. In view of this possible effect, and the fact that no presupposed correction can be made with confidence for charge repulsion, the work of Kurtz (19) should be extended to determine the validity of his conclusion of an inherent, strong inhibitor effect for the naphthalene nucleus.

The fact that the remaining indolecarboxylate ions are subject to apparent charge repulsions of 0.5 to 0.7 Kcal/Mole can have one of several explanations. One such possibility is the presence of secondary repulsions, due to more distant negative charges in the region of the active site. Another is the lack of shielding of the more distant indole positions, against the charge near the indole nitrogen. The latter explanation requires that the negative charge not be coplanar with the indole ring, so as to avoid significant π electron shielding, and also that the group responsible for the steric repulsions at the 4 position is not situated so as to increase the microscopic dielectric constant between the indole nitrogen and the 4 position. While it is tempting to use $\delta(-\Delta F^{o})$ data to fix the position of the negative charge relative to the indole ring, lack of knowledge of microscopic dielectric constants in the region of the active site makes such determinations unreliable.

The residual $\delta(-\Delta F^{o})$ of charge repulsion might also be due to a decrease in the degree of hydration of the carboxylate ions, with a corresponding energy change associated with removal of the solvent molecules from the anions. Benjamin and Gold (51) have found that $\Delta F^{o}_{hydr.}$ for halide ions were of the order of -0.3 Kcal/Mole and -0.8 Kcal/Mole for $S^{=}_{2}$. Thus, such a possibility might easily account for the free energy changes observed in indole carboxylate ions 3 through 6.

It is of interest to try to rationalize published data for other pairs of carboxylate ions and amides on the basis of the hypothesis of a negative group near the indole nitrogen, in its complex with the active site. These data are given in table 3.

There appears to be a strong charge repulsion which decreases as the center of negative charge is removed from the aromatic nucleus. Even considering the effect of the greater pH, it is not clear why the effect for benzoate should exceed the maximum effect observed for the indole-carboxylate ions, since, due to the symmetry of the benzene ring, benzoate should be able to position itself on the active site so as to minimize repulsions. It should be noted, however, that this symmetry gives rise to statistical considerations. Benzamide, which is subject only to steric repulsions, will have more stable active site configurations available to it than benzoate, which is subject to both steric and electrostatic repulsions. As the side chain length of an aromatic inhibitor is increased, interpretation of negative charge effects becomes more difficult because of the possibility of multifunctional binding.

Table 3: The Inhibition Constants of Various Pairs of Charged and Uncharged Species at pH 7.9.

Ref.	Inhibitor	K_I [a]	$\frac{K_{I_{COO^-}}}{K_{I_{CONH_2}}}$	$\delta(-\Delta F^o)^b_{COO^- \; CONH_2^-}$
16	benzoate	150	15	-2.4
"	benzamide	10		
"	phenylacetate	200	13.3	-1.7
"	phenylacetamide	15		
"	phenylpropionate	25	3.6	-0.8
"	phenylpropionamide	7		
"	phenylbutyrate	60	5	-1.0
"	phenylbutyramide	12		
"	β-(β-indole)propionate	15	6.5	-1.1
"	β-(β-indole)propionamide	2.3		
18	acetyl-D-tryptophanate	7.5	3.3	-0.7
"	acetyl-D-tryptophanamide	2.3		

a) in units of 10^{-3} M

b) in units of Kcal/Mole at 25.0° C

Conclusion:

A study of the variation of the inhibition constants of indolecarboxylate ions and indolecarboxamides, with the position of substitution, reveals steric repulsion, in excess of 1 Kcal/Mole, toward the 4 substituted derivatives and electrostatic repulsions of the same magnitude toward indole-2- and -7-carboxylate ions. This clearly indicates that indole complexes with the active site of α-chymotrypsin in a preferred orientation, with a negatively charged group situated near the indole nitrogen atom and a neutral group near the 4-position.

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Appendix I.

α-Chymotrypsin Catalysed Hydrolyses of N-Acetyl-L-tyrosinhydroxamide at pH 7.60, 25.0° C and 0.20 M NaCl.

$(S)_o$ [a]	Inhibitor	$(I)_o$ [b]	$(E)_o$ [c]	v_o [d]
0.513			2.11	6.08
"			"	5.52
"			2.18	6.62
0.580			2.06	6.36
"			"	6.43
0.770			2.11	8.59
"			2.18	8.78
1.026			2.11	10.28
"			2.18	11.11
1.161			2.06	11.31
"			"	11.58
1.741			"	16.57
"			"	16.28
1.865			2.11	16.65
2.052			2.18	19.71
2.322			2.06	20.21
"			"	21.22
3.078			2.11	22.28e
"			2.18	27.65e
3.482			2.06	26.28
"			2.06	25.95
4.104			2.18	29.36
"			"	30.43
4.643			2.06	28.87
"			"	30.28
0.514	Indole	1.81	1.51	1.11
"	"	"	"	1.19
0.515	"	"	2.20	1.60
"	"	"	"	1.73
0.770	"	"	1.51	1.80
"	"	"	"	1.72
0.773	"	"	2.20	2.22
"	"	"	"	2.30
1.027	"	"	1.51	2.42
"	"	"	"	2.36
1.030	"	"	2.20	3.01
"	"	"	"	2.99
2.059	"	"	2.16	5.97
"	"	"	"	6.18
2.060	"	"	2.20	5.51
"	"	"	"	4.98

$(S)_o$ [a]	Inhibitor	$(I)_o$ [b]	$(E)_o$ [c]	v_o [d]
3.089	Indole	1.81	2.16	7.76
"	"	"	"	7.71
3.090	"	"	2.20	7.24
"	"	"	"	6.91
3.604	"	"	2.16	9.72
"	"	"	"	9.35
0.617	"	3.44	2.09	1.04
"	"	"	"	0.84
0.925	"	"	"	1.39
"	"	"	"	1.40
1.039	"	"	2.11	1.88
"	"	"	"	1.79
1.233	"	"	2.09	1.68
"	"	"	"	1.81
2.078	"	"	2.11	3.38
"	"	"	"	3.77
2.466	"	"	2.09	4.21
"	"	"	"	3.11
3.083	"	"	"	4.91
"	"	"	"	4.78
3.117	"	"	2.11	5.06
"	"	"	"	5.30
3.699	"	"	2.09	5.55
"	"	"	"	5.23
4.156	"	"	2.11	7.27
"	"	"	"	6.18
5.195	"	"	"	8.07
"	"	"	"	9.15
6.234	"	"	"	8.01
"	"	"	"	8.77
1.026	Indole-2-carboxylate	2.25	2.10	7.31
"	"	"	"	7.44
2.052	"	"	"	13.86
"	"	"	"	13.94
3.078	"	"	"	18.85
"	"	"	"	19.00
4.104	"	"	"	20.93
"	"	"	"	21.88
5.130	"	"	"	25.76
"	"	"	"	25.23
7.182	"	"	"	26.79e
"	"	"	"	30.86
1.026	"	7.54	2.20	4.48
"	"	"	"	4.71
2.052	"	"	"	6.96e
"	"	"	"	8.59

$(S)_o$ [a]	Inhibitor	$(I)_o$ [b]	$(E)_o$ [c]	v_o [d]
3.078	Indole-2-carboxylate	7.54	2.20	11.65
"	"	"	"	12.51
4.104	"	"	"	14.62
"	"	"	"	15.53
5.130	"	"	"	18.68
6.156	"	"	"	21.27
"	"	"	"	23.87e
0.879	"	4.50	2.06	5.17
"	"	"	"	5.30
1.758	"	"	"	12.81e
"	"	"	"	9.43
2.637	"	"	"	12.59
"	"	"	"	13.83
3.516	"	"	"	15.82
"	"	"	"	15.77
4.395	"	"	"	18.77
"	"	"	"	18.05
5.274	"	"	"	22.67e
"	"	"	"	18.89
1.025	Indole-2-carboxamide	0.522	2.15	7.28
"	"	"	"	7.82
2.050	"	"	"	12.47
"	"	"	"	13.02
3.075	"	"	"	16.38
"	"	"	"	17.04
4.100	"	"	"	19.38
"	"	"	"	22.16
5.125	"	"	"	24.11
"	"	"	"	26.48
6.150	"	"	"	28.67
"	"	"	"	27.90
1.030	"	1.044	2.11	4.76
1.545	"	"	"	6.65
2.060	"	"	"	8.52
"	"	"	"	9.33
2.575	"	"	"	9.95
"	"	"	"	11.21
3.090	"	"	"	12.19
"	"	"	"	12.49
4.120	"	"	"	15.40
"	"	"	"	16.39
0.881	Indole-3-carboxylate	4.62	2.18	6.41
1.762	"	"	"	11.16
"	"	"	"	12.30
2.643	"	"	"	13.95
3.524	"	"	"	19.25

$(S)_o$ [a]	Inhibitor	$(I)_o$ [b]	$(E)_o$ [c]	v_o [d]
4.405	Indole-3-carboxylate	4.62	2.18	21.87
"	"	"	"	22.64
5.286	"	"	"	24.84
"	"	"	"	23.27
0.799	"	6.72	2.14	4.16
"	"	"	"	3.84
1.598	"	"	"	8.16
"	"	"	"	7.29
2.397	"	"	"	11.24
"	"	"	"	9.64
3.195	"	"	"	14.21
"	"	"	"	13.62
3.995	"	"	"	15.74
"	"	"	"	15.61
"	"	"	"	15.26
0.585	"	5.12	2.19	3.24
"	"	"	"	3.23
1.170	"	"	"	5.94
1.755	"	"	"	8.42
"	"	"	"	8.73
2.340	"	"	"	10.58
"	"	"	"	11.07
2.295	"	"	"	13.06
"	"	"	"	13.07
3.510	"	"	"	15.07
"	"	"	"	15.00
0.88	Indole-3-carboxamide	1.89	2.06	4.31
"	"	"	"	4.12
1.76	"	"	"	7.08
"	"	"	"	7.39
2.64	"	"	"	9.86
"	"	"	"	9.47
3.52	"	"	"	12.38
"	"	"	"	12.38
4.40	"	"	"	14.25
5.28	"	"	"	16.92
"	"	"	"	16.45
0.582	"	2.02	2.20	3.11
"	"	"	"	2.99
1.164	"	"	"	6.15
"	"	"	"	5.53
1.746	"	"	"	8.60
"	"	"	"	7.68
2.328	"	"	"	10.38
"	"	"	"	9.74
2.910	"	"	"	12.57
"	"	"	"	12.22

$(S)_o$ [a]	Inhibitor	$(I)_o$ [b]	$(E)_o$ [c]	v_o [d]
3.492	Indole-3-carboxamide	2.02	2.20	14.94
"	"	"	"	13.95
0.588	"	3.01	2.09	2.10
"	"	"	"	2.07
1.175	"	"	"	3.80
"	"	"	"	3.91
1.763	"	"	"	5.58
"	"	"	"	6.09
2.350	"	"	"	7.43
"	"	"	"	7.02
2.938	"	"	"	9.16
"	"	"	"	8.64
3.525	"	"	"	9.62
"	"	"	"	9.34
0.585	Indole-4-carboxylate	3.98	2.18	5.00e
"	"	"	"	4.58e
1.170	"	"	"	9.34
"	"	"	"	9.36
1.754	"	"	"	12.98
2.339	"	"	"	15.94
"	"	"	"	16.00
2.924	"	"	"	17.92e
"	"	"	"	18.42
3.509	"	"	"	20.83
"	"	"	"	20.77
0.584	"	13.32	2.14	3.04
"	"	"	"	2.93
1.167	"	"	"	6.16
"	"	"	"	5.71
1.751	"	"	"	8.71
"	"	"	"	8.19
2.334	"	"	"	11.73e
"	"	"	"	10.72
2.918	"	"	"	12.83
"	"	"	"	12.33
3.502	"	"	"	14.11
"	"	"	"	14.12
0.586	Indole-4-carboxamide	1.97	2.06	4.71
"	"	"	"	4.64
1.172	"	"	"	8.58
"	"	"	"	8.41
1.758	"	"	"	11.63
"	"	"	"	11.93
2.344	"	"	"	14.23
"	"	"	"	14.68

$(S)_o$ [a]	Inhibitor	$(I)_o$ [b]	$(E)_o$ [c]	v_o [d]
2.930	Indole-4-carboxamide	1.97	2.06	17.68
"	"	"	"	16.60e
3.516	"	"	"	19.82
"	"	"	"	19.34
0.583	"	6.07	2.07	2.73
"	"	"	"	2.80
1.167	"	"	"	5.11
"	"	"	"	5.40
1.750	"	"	"	7.61
"	"	"	"	7.45
2.333	"	"	"	9.62
"	"	"	"	9.57
2.917	"	"	"	11.64
3.500	"	"	"	13.20
"	"	"	"	13.32
0.581	Indole-5-carboxylate	10.72	2.17	1.27e
"	"	"	"	1.38
1.162	"	"	"	2.72
1.742	"	"	"	3.99
"	"	"	"	3.60e
2.323	"	"	"	5.19
"	"	"	"	5.20
2.904	"	"	"	6.34
"	"	"	"	6.25e
3.485	"	"	"	6.49e
"	"	"	"	7.58
0.583	"	4.02	2.18	3.06
"	"	"	"	3.14
1.167	"	"	"	5.84
"	"	"	"	5.79
1.750	"	"	"	8.23
"	"	"	"	8.45
2.334	"	"	"	10.72
"	"	"	"	10.44
2.917	"	"	"	12.47
"	"	"	"	12.48
3.500	"	"	"	14.22
"	"	"	"	14.30
0.586	Indole-5-carboxamide	2.03	2.05	2.66
"	"	"	"	2.68
1.172	"	"	"	4.89
"	"	"	"	5.11
1.757	"	"	"	6.98
2.343	"	"	"	7.07
"	"	"	"	7.07

$(S)_o$ [a]	Inhibitor	$(I)_o$ [b]	$(E)_o$ [c]	v_o [d]
2.929	Indole-5-carboxamide	2.03	2.05	10.79
"	"	"	"	10.45
3.515	"	"	"	12.25
"	"	"	"	11.74
0.585	"	1.40	2.02	3.13
"	"	"	"	3.26
1.170	"	"	"	6.06
"	"	"	"	6.19
1.755	"	"	"	8.05
"	"	"	"	8.69
2.304	"	"	"	11.03
"	"	"	"	10.92
2.926	"	"	"	12.95
"	"	"	"	12.48
3.511	"	"	"	14.29
"	"	"	"	13.99
0.508	Indole-6-carboxylate	4.05	2.03	1.60
"	"	"	"	1.63
1.016	"	"	"	3.21
"	"	"	"	3.04
1.523	"	"	"	4.24
"	"	"	"	4.44
2.031	"	"	"	5.84
"	"	"	"	5.83
2.539	"	"	"	7.14
"	"	"	"	6.88
3.053	"	"	"	8.36
"	"	"	"	7.89
0.586	"	1.97	2.06	2.95
"	"	"	"	2.90
1.171	"	"	"	5.39
"	"	"	"	5.52
1.757	"	"	"	7.83
"	"	"	"	7.92
2.342	"	"	"	10.09
"	"	"	"	10.07
2.928	"	"	"	11.90
"	"	"	"	11.99
3.514	"	"	"	13.48
"	"	"	"	14.02
0.586	Indole-6-carboxamide	2.01	2.15	1.69
"	"	"	"	1.62
1.171	"	"	"	3.26
"	"	"	"	3.32
1.757	"	"	"	4.61
"	"	"	"	4.55

$(S)_o$ [a]	Inhibitor	$(I)_o$ [b]	$(E)_o$ [c]	v_o [d]
2.342	Indole-6-carboxamide	2.01	2.15	6.19
"	"	"	"	6.09
2.928	"	"	"	7.34
"	"	"	"	7.39
3.514	"	"	"	8.63
"	"	"	"	8.39
0.586	"	0.981	2.07	2.60
"	"	"	"	2.61
1.173	"	"	"	5.07
"	"	"	"	4.86
1.759	"	"	"	7.10
"	"	"	"	7.14
2.345	"	"	"	8.86
"	"	"	"	9.22
2.932	"	"	"	10.95
"	"	"	"	10.55
3.518	"	"	"	12.33
"	"	"	"	17.58e
0.583	Indole-7-carboxylate	4.06	2.09	4.58
"	"	"	"	4.50
1.166	"	"	"	8.71
"	"	"	"	8.31
1.749	"	"	"	11.76
"	"	"	"	11.87
2.332	"	"	"	14.62
2.916	"	"	"	17.47
"	"	"	"	17.17
3.499	"	"	"	18.82
"	"	"	"	18.97
0.594	"	10.79	2.05	3.01
"	"	"	"	2.95
1.187	"	"	"	5.53
"	"	"	"	5.74
1.781	"	"	"	7.80
"	"	"	"	8.25
2.374	"	"	"	10.19
2.968	"	"	"	12.26
"	"	"	"	11.79
3.562	"	"	"	13.92
"	"	"	"	13.64
0.582	Indole-7-carboxamide	0.975	2.20	3.74
"	"	"	"	3.68
1.165	"	"	"	6.55
"	"	"	"	6.57
1.747	"	"	"	9.68
"	"	"	"	9.54

$(S)_o$ [a]	Inhibitor	$(I)_o$ [b]	$(E)_o$ [c]	v_o [d]
2.329	Indole-7-carboxamide	0.975	2.20	12.35
"	"	"	"	11.66
2.912	"	"	"	14.33
"	"	"	"	14.02
3.494	"	"	"	16.60
"	"	"	"	16.00

a) in units of 10^{-2} M

b) in units of 10^{-3} M

c) in units of 10^{-2} mg. P.N./ml.

d) in units of 10^{-5} M/ℓ min.

e) pt. excluded by statistical test

Appendix II.

Inhibition Constants of Indole Derivatives at pH 7.60, 25.0°C and 0.20 M NaCl

Inhibitor	$(I)_o$ [a]	k_3 [b]	K_I [a]
indole	1.81	14.1±2.4	0.63±0.10
"	3.44	70±86	0.49±0.06
indole-2-carboxylate	2.25	29.6±1.7	4.5±1.4
"	7.54	34.5±4.5	4.2±0.7
"	4.50	21.3±1.7	5.7±1.6
indole-2-carboxamide	0.522	30.4±3.4	0.79±0.28
"	1.044	35.7±9.3	0.64±0.13
indole-3-carboxylate	4.62	25.4±3.0	7.6±2.8
"	6.72	28.3±6.3	4.9±1.1
"	5.12	25.9±1.6	4.0±0.7
indole-3-carboxamide	1.89	20.3±1.6	1.30±0.25
"	2.02	23.9±3.7	1.37±0.25
"	3.01	17.4±2.6	1.24±0.20
indole-4-carboxylate	3.98	24.4±0.3	11.4±3.8
"	13.32	25.1±2.7	10.2±2.0
indole-4-carboxamide	1.97	26.9±1.0	4.5±1.4
"	6.07	27.1±1.6	4.0±0.7
indole-5-carboxylate	10.72	30.9±2.2	2.6±0.3
"	4.02	23.6±0.9	3.0±0.5
indole-5-carboxamide	2.03	20.1±1.3	1.33±0.22
"	1.40	22.8±1.8	1.35±0.27
indole-6-carboxylate	4.05	22.6±3.6	1.48±0.17
"	1.97	26.3±1.2	1.44±0.23
indole-6-carboxamide	2.01	22.8±2.5	0.60±0.08
"	0.981	23.5±2.5	0.60±0.09
indole-7-carboxylate	4.06	25.1±1.1	9.0±2.8
"	10.79	24.6±1.6	8.2±1.4
indole-7-carboxamide	0.975	24.2±1.7	0.98±0.14

a) in units of 10^{-3} M

b) in units of 10^{-3} Mole/mg. P.N. min.

PART II

STERIC REQUIREMENTS FOR SUBSTRATES OF α-CHYMOTRYPSIN

Introduction:

The presence of aliphatic groups β to the carboxyl group causes marked decreases in the rates of acid catalysed esterifications of acids (1, 2) and the acid (3, 4) and base catalysed (5-7) hydrolyses of esters and amides (8). Detailed analysis (9) of these data indicates that this effect is steric, rather than electronic, in nature. Since the effects observed for single β substituents are greater than effects for single α substituents and since γ substituents exert noticeable steric effects only in cases of α, γ or β, γ disubstitution, these effects are taken as evidence for a coiled structure for carboxyl derivatives in solution (10). Esterifications and hydrolyses, with the exception of hydrolyses in conc. H_2SO_4, have been shown to proceed through a tetrahedral transition state (11), probably by the approach of reactant in a direction perpendicular to the plane of the carbonyl group. Atoms situated six bonds from the carbonyl oxygen could present large steric repulsions toward the approach of reactants and the formation of tetrahedral intermediates, thus causing large decreases in rates of reactions that proceed by such a mechanism. This effect has been qualitatively formulated in Newman's "rule of six" (12): "In reactions involving addition to an unsaturated function, the greater the number of atoms in the six position, the greater will be the steric effect." The six position is defined as six atoms from the further unsaturated atom (fig. 1).

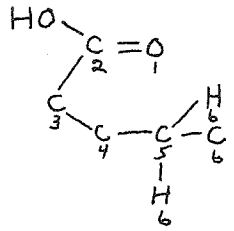


Figure 1

Consideration of the variations of the kinetic constants associated with α-chymotrypsin catalysed hydrolyses of amino acid derivatives with increasing β substitution could yield important information about the mechanism of enzyme action. If increasing β substitution in a particular series of amino acid derivatives causes a decrease in k_3, the apparent first order rate constant for the rate determining decomposition of the enzyme-substrate complex, important mechanistic conclusions could be drawn. The amino acid side chain of the substrate in the complex would have to be coiled so as to cause large interactions between its six position atoms and the carbonyl group and the transition state for the decomposition of the complex would contain a tetrahedral carbonyl carbon. Insensitivity of k_3 to β steric effects would probably indicate that either the side chain in the complex was uncoiled or that the complex decomposition transition state does not resemble the transition states for acid or base catalysed hydrolyses.

Theoretical interpretation of the effect of β-steric hindrance on K_s, the apparent dissociation constant of the enzyme substrate complex, is more difficult, due to the uncertainty of the exact nature of this constant. If K_s approximates a true equilibrium constant, i.e., k_3 is

small compared to k_2, the rate of decomposition to reactants, then K_s will be independent of the energy barrier for formation and decomposition. Any observed variations in K_s with the degree of β substitution would be due to specific interactions between the substrates and the enzyme surface.

If K_s is not an equilibrium constant, the effect on two transition states, for decomposition to reactants as well as to products, must be considered. Interpretation must then be based on a number of assumptions as to the nature of the complex as well as its transition states of decomposition and theoretical conclusions based on these assumptions would necessarily be questionable. K_s can still be increased by internal steric hindrance due to repulsions between groups in the substrate, preventing proper orientation of the substrate for binding to the enzyme surface, even though K_s is not an equilibrium constant.

While there are few data on the effect of increasing β steric hindrance on chymotrypsin catalysed hydrolyses, a marked suppression of rates has been observed in the series: N-acetyl-L-leucine, L-isoleucine and L-valine methyl esters (13). Due to the presence of "wall effect" (14) errors at the low enzyme concentrations required for the first substrate, no comparison of kinetic constants has been made. It was considered useful to examine more highly branched substrates for comparison with the extensive data for L-valine derivatives. In particular, three amino acids: t-leucine (2-amino-3,3-dimethylbutyric acid), β,β-dimethylphenylalanine and 2,6-dimethyltyrosine were considered.

t-Leucine represents a member of a directly comparable series of amino acids which includes the naturally occurring acids alanine, α-aminobutyric acid, valine, leucine and isoleucine. A study of this series, with its wide variety of degrees of branching at the β position, is a necessary precursor to a structural model for the enzyme substrate complex.

β,β-Dimethylphenylalanine is useful for comparison with aliphatic and aromatic amino acids. Since aromatic and other cyclic groups situated β to the carboxyl group increase the enzymatic susceptibility of amino acid derivatives (15,16), the phenyl group might bind with the active site so as to effect the orientation of the methyl groups and minimize steric hindrance. On the other hand, the methyl groups might, by interaction with the ortho-ring hydrogens, prevent proper orientation of the phenyl ring. The ring could then exert a steric deactivating effect and the k_3's of β,β-dimethylphenylalanine derivatives would fall not only below those of the corresponding phenylalanine derivatives but might also be less than the corresponding valine derivatives.

The methyl groups in 2,6-dimethyltyrosine would not directly interfere with the carboxyl group since molecular models show that such interaction is of the order of that for the γ-methyl groups in leucine. This molecule should, however, show internal steric effects, due to interaction between the β hydrogens and the ortho methyl groups, approximating those in β,β-dimethylphenylalanine.

The recent discovery that D-1-keto-3-carbomethoxy-1, 2, 3, 4-tetrahydroisoquinoline (17) is a much better substrate than its "natural" L-antipode and is comparable, in rates of enzyme catalysed hydrolyses, to N-benzoyl-L-phenylalanine methyl ester, has stimulated interest in aromatic amino acids containing a bridge between the amine and aromatic groups. Because of its availablity from synthetic schemes used in work currently in progress (18), methyl indole-2-carboxylate was studied as part of a general investigation of such cyclic acids.

Summary:

N-Acetyl-D,L-t-leucine, -β,β-dimethylphenylalanine, -2,6-dimethyltyrosine methyl esters and methyl indole-2-carboxylate were synthesized. The synthesis of 2,6-dimethyltyrosine represents an original synthesis of a previously unreported amino acid. α-Chymotrypsin shows no catalytic activity in the hydrolysis of N-acetyl-D,L-t-leucine methyl ester, N-acetyl-D,L-β,β-dimethylphenylalanine methyl ester or methyl indole-2-carboxylate. The enzyme has a slight catalytic activity towards the hydrolysis of N-acetyl-D,L-2,6-dimethyltyrosine methyl ester. The enzymatic hydrolysis was shown to be stereospecific for the d-ester. Resolution of t-leucine was achieved by fractional crystallization of the brucine salts of N-formyl-D,L-t-leucine. Resolution of N-acetyl-D,L-β,β-dimethylphenylalanine was achieved by fractional crystallization of the ℓ-α-phenylethylamine salts.

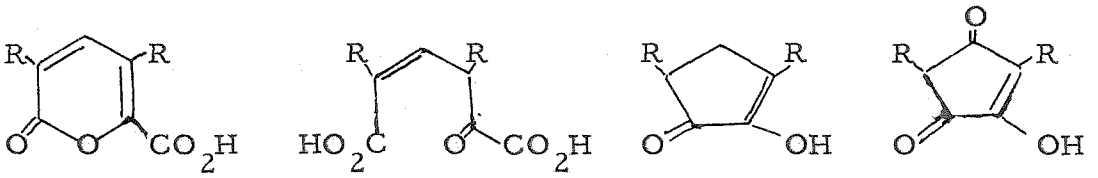
Synthesis:

t-Leucine (pseudoleucine) was prepared by the method of Knoop and Landmann (19). This scheme involves the alkaline permanganate oxidation of pinacalone to trimethylpyruvic acid and formation and reduction of the oxime. The ready decomposition of 2-oximino-3,3-dimethybutyric acid to pivalonitrile, on mild heating, was the only difficulty not described in previous syntheses. After discouraging results with catalytic and amalgam reductions of the oxime, the previously described (19) zinc-acetic acid reduction was found to be satisfactory.

Jönnson (20) has reported the failure of the synthesis of β,β-dimethylphenylalanine (neophenylalanine) from α,α-dimethylphenylacetaldehyde but does describe a successful synthesis based on permanganate oxidation of 4,6-di-(α,α-dimethylbenzyl)pyrogallol to α-keto-β-phenylisovaleric acid and reduction of the corresponding oximino acid. Attempts to repeat the oxidation step of this scheme were unsuccessful. Contrary to Jönnson's report, the pyrogallol derivative did not dissolve readily in 0.8 N NaOH. The reaction was carried out in neutral aqueous acetone. The bicarbonate soluble products were isolated by Jönnson's procedure. On attempted fractional distillation of the esterified mixture at a pot temperature of 170° and a pressure of 7 mm., extensive decomposition occurred without any distillate being collected.

Campbell (21) has reported that air oxidation of 4,6-di-t-butylpyrogallol in alkaline methanol gave four products:

3,5-di-t-butylcoumalic acid (I), 3,5-di-t-butyl-2-ketohexene-4-dioic acid (II), 3,5-di-t-butylcyclopentadione-1,2 (III) and 3,5-di-t-butylcyclopentatrione (IV) in equal amounts.



R = $C(CH_3)_3$

I II III IV

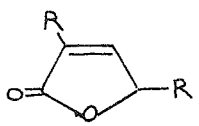
R = $C(CH_3)_2C_6H_5$

V VI VII VIII

Since the low yields and the side products (22) reported for the permanganate oxidation of the pyrogallol derivative indicate a very complex reaction system, air oxidation of the pyrogallol derivative was attempted. It was hoped that products analogous to those described by Campbell could be cleanly oxidized to the desired α-keto-β-phenylisovaleric acid. Structures V and VI were considered particularly likely intermediates for the synthesis of the keto acid.

On reaction of an alkaline methanolic solution of 4,6-di(α,α-dimethylbenzyl)pyrogallol with oxygen, two products were isolated and identified. When recrystallization of the acidic products was attempted from hexane, an apparent chemical reaction occurred and V, m.p. 149.0-149.5°, precipitated from the hot solvent. This product was probably formed, in part, by lactonization of VI. The ease of lactonization varied among several experiments and, in one case, VI, m.p.

116.0-118.5°, was isolated instead of V. These products were isolated in 50-60% yields. A small amount of neutral product (IX), m.p. 134-135°, was isolated and proved to be the lactone of 2,4-di(α,α-dimethylbenzyl)-4-hydroxycrotonic acid. Infrared analysis of impure neutral product mixtures showed that this compound was present in large amounts.



$R = C_6H_5C(CH_3)_2-$

IX

The assignment of the structure of V was based on the following evidence: The analysis and the molecular weight fit the formula $C_{24}H_{24}O_4$. The material was soluble in aqueous bicarbonate and neutralization and saponification equivalents indicate the presence of one free carboxyl group and one ester or lactone group. The material did not react with 2,4-dinitrophenylhydrazine and gave no color reaction with $FeCl_3$. A solution of the material in 5% NaOH has a maximum absorption in the ultraviolet region at 3050 Å, which disappeared if the solution was allowed to stand overnight. In methanolic solutions, this band appeared at 3030Å (ϵ_{max} = 8650) (23). The infrared spectrum in $CHCl_3$ had an intense, broad carbonyl peak from 1745-1705 cm^{-1} which is probably composed of unresolved lactone and acid bands. Oxidation in alkaline permanganate gave a 60% yield (based on two moles of product per mole of V) of α-keto-β-phenylisovaleric acid (isolated as the oxime), a 5% yield of IX and oxalic acid.

Compound VI was identified by comparison with the product of saponification of V. The melting point of a mixture of these two materials was not depressed. Oxidation of VI by alkaline permanganate gave the same products as V.

The structure of IX was assigned on the basis of the following evidence: Analysis indicated a formula of $C_{22}H_{24}O_2$ or $C_{23}H_{26}O_2$. The material had an intense infrared absorption band at 1755 cm^{-1}. The ultraviolet spectrum indicated only phenyl absorption. The compound was insoluble in aqueous sodium bicarbonate $NaHCO_3$, 5% NaOH and 85% H_3PO_4 but soluble in conc. H_2SO_4. It gave no precipitate with 2,4-dinitrophenylhydrazine, no color with $FeCl_3$, and did not decolorize solutions of Br_2 in CCl_4 or $KMnO_4$. If the compound was heated in ethanolic hydrolxylamine at reflux temperature for five hours, the product gave no color with $FeCl_3$. However, infrared analysis of the product showed that lactone hydrolysis had occurred. The carbonyl peak of the hydrolysate was shifted to 1705 cm^{-1} and an OH absorption had appeared at 3500 cm^{-1}. Partition of the hydrolysate between aqueous bicarbonate and ether did not cause any change in the infrared spectra of either fraction. The nuclear magnetic resonance spectrum (fig. 2) (24) showed the presence of aromatic protons, two types of methyl, and one type each of methine and vinyl protons, in the approximate ratios 12:10:1:1. The methine and vinyl protons appeared as doublets. The methyl protons resolved into doublets at slow sweep speeds.

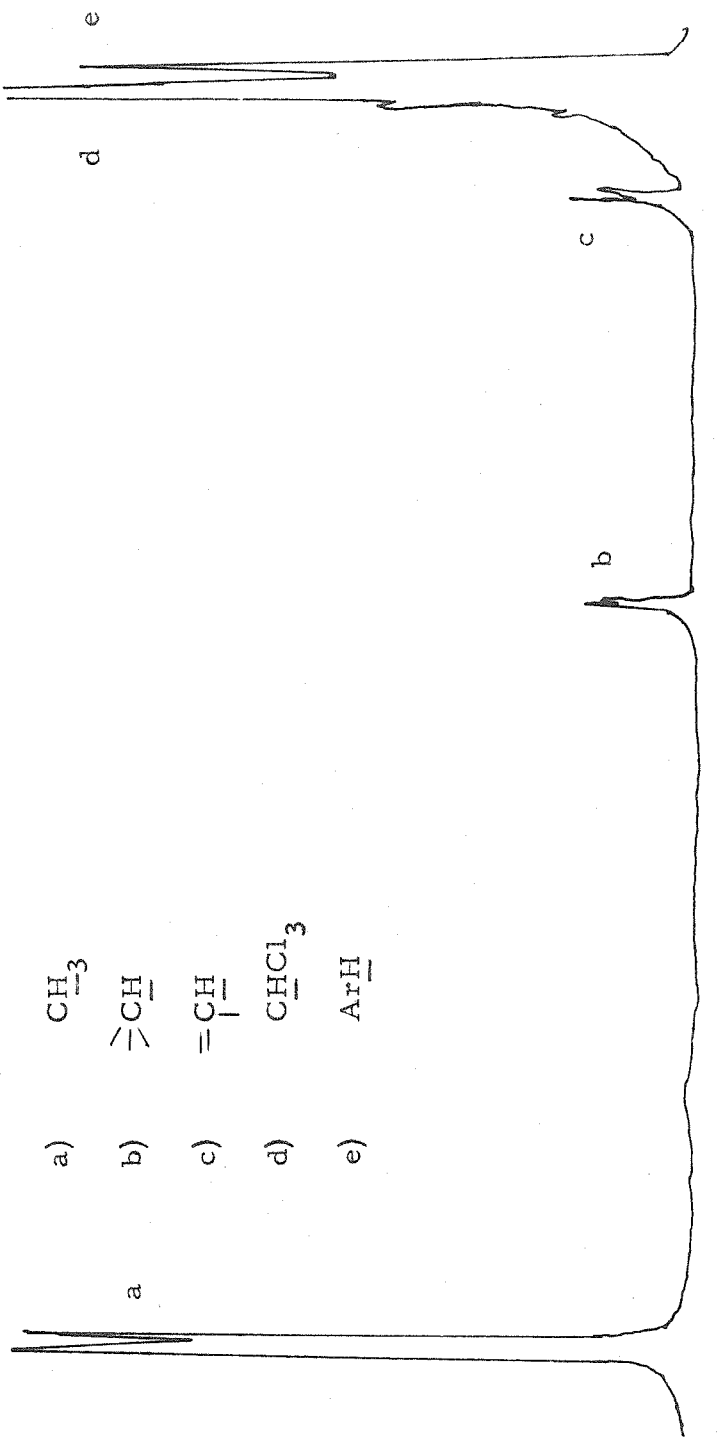


Figure 2. Nuclear magnetic resonance spectrum of IX in $CHCl_3$.

The infrared and ultraviolet spectra are in good agreement with those of model compounds in the literature, in particular the lactone of 2-methyl-4-hydroxy-4-phenylcrotonic acid (25). The double bond was assigned the α,β rather than the β,γ position because of the carbonyl absorption frequency and the lack of either a positive $FeCl_3$ test or a ketone absorption by the hydrolysate. The anomalous features of this compound appear to be due to steric hindrance. The resistance of the unconjugated double bond to oxidation and bromination is similar to the effect observed by Jönsson for the resistance to periodate oxidation of 2,4-di-(α,α-dimethylbenzyl)-4-carboxy-2,3-dihydroxybutyrolactone (22) and by Bartlett and Woodward (26) for the resistance to permanganate oxidation of 1,1-dineopentylethylene. It has been suggested (27) that the resolution of the nuclear magnetic resonance peaks of the quaternary methyls into doublets is a result of hindered rotation of the dimethylbenzyl groups about the bonds linking them to the lactone ring.

The alkaline permanganate oxidation of V provided a satisfactory synthetic route to α-oximino-β-phenylisovaleric acid. The yield for this scheme was comparable to the yield for direct oxidation of the pyrogallol derivative, as reported by Jönnson, and the isolation of product, especially for small scale reactions, was less tedious. After several discouraging results with $SnCl_2$ and catalytic reduction of the oximino acid, the sodium amalgam reduction of Jönnson (28) was carried out in 40-50% yield. In one run a nitrogen free by-product, m.p. 184-186°, was isolated in 25% yield. This material is acidic and gives

negative tests with $FeCl_3$ and 2,4-dinitrophenylhydrazine. A saturated $CHCl_3$ solution of the material showed absorption maxima at 3420, 3010, 1792 and 1710 cm^{-1}.

2,6-Dimethyltyrosine, m.p. 230-231° (decomp.), was prepared by Sommelet chloromethylation (29) of O-carbethoxy-3,5-dimethylphenol, reaction of the resulting benzyl chloride with diethyl acetamidomalonate and decomposition of the product, diethyl acetamido-(4-hydroxy-2,6-dimethylbenzyl)-malonate, with HBr to obtain the amino acid. If the reaction time of the chloromethylation reaction was extended, 2,4,6-trichloromethyl-3,5-dimethylphenol, m.p. 144-147°, was obtained. At shorter reaction times, the monochloromethyl product described by Sommelet, b.p. $_{3mm.}$ 145°, was obtained. The structure assignment of Sommelet was verified by the nuclear magnetic resonance spectrum (fig. 3), which showed one type of aromatic proton, singlet methyls, singlet methylenes, quadruplet methylenes and triplet methyls in the ratios 2:6:2:2:3. The appearance of only one type of aromatic protons and aromatic substituted methyls is consistent only with p-substitution.

Methyl indole-2-carboxylate was prepared by the Rydon and Tweddle (30) modification of the Fischer (31) indole synthesis.

Preliminary Kinetic Studies:

The hydrolyses of N-acetyl-β,β-dimethylphenylalanine methyl ester and N-acetyl-D,L-t-leucine methyl ester were not appreciably

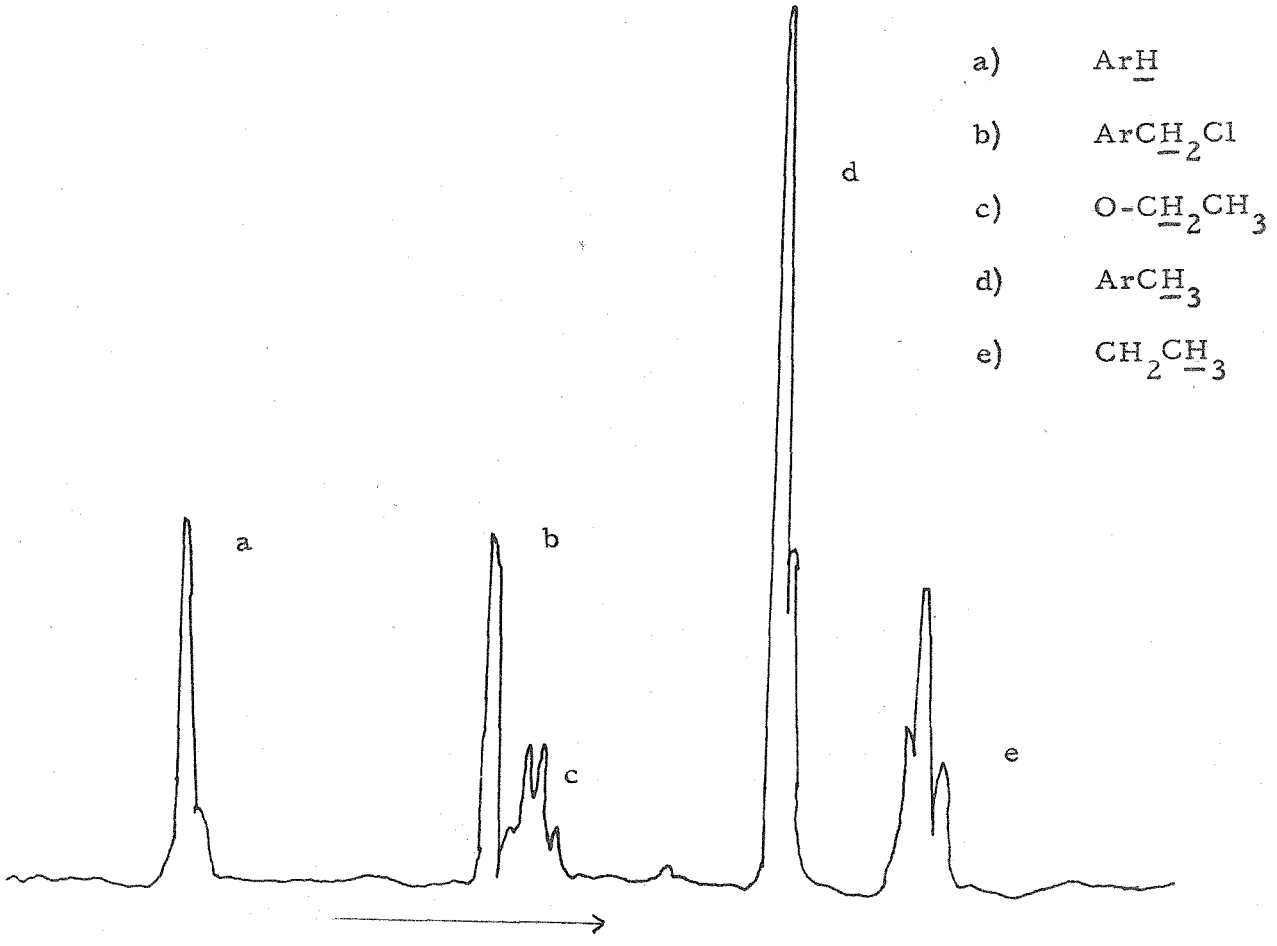


Figure 3. Nuclear magnetic resonance spectrum of O-carbethoxy-4-chloromethyl-3,5-dimethylphenol in CCl_4.

catalysed by α-chymotrypsin at pH 7.90, 25.0° , 0.20 M NaCl and an enzyme concentration of 1.0 mg. protein nitrogen/ml. Due to the low water solubility of N-acetyl-D,L-2,6-dimethyltyrosine methyl ester, it was necessary to study the kinetics of hydrolysis of this compound in aqueous methanol and acetone solutions. This compound was slowly hydrolysed by high concentrations of α-chymotrypsin to 50% conversion (see fig. 4). Rates in aqueous acetone were faster than rates in aqueous methanol and increasing concentrations of non-aqueous solvent components decreased the rates of enzyme catalysed hydrolysis. The data for this substrate are summarized in table I.

Methyl indole-2-carboxylate was extremely insoluble in water and it was necessary to study the kinetics of hydrolysis in systems containing high concentrations of non-aqueous components. In 25% aqueous dioxane at pH 7.90, 0.20 M NaCl and 25.0° C, the rate of production of acid for the system containing $2.16 \cdot 10^{-3}$ M methyl indole-2-carboxylate and 0.20 mg. P.N./ml. of α-chymotrypsin was one half the rate of production of acid for the system containing enzyme only. This indicates that methyl indole-2-carboxylate acts as a competitive inhibitor to the "enzyme blank" rather than as a substrate.

Resolution Studies:

t-Leucine was resolved by fractional crystallization of the brucine salts of N-formyl-D,L-t-leucine (32). There is controversy about the absolute configuration of the antipodes. Abderhalden (32) has

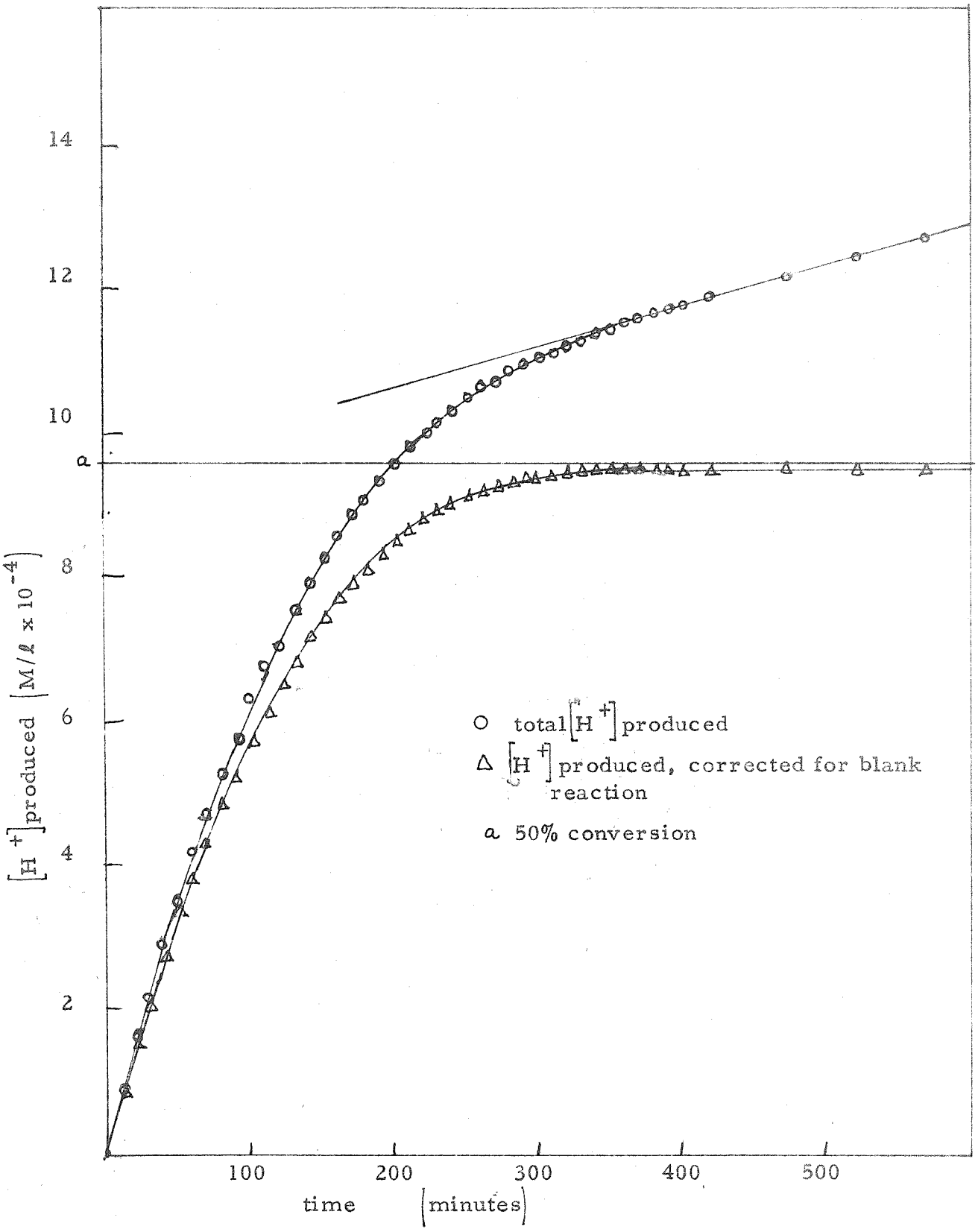


Figure 4. Hydrolysis of N-acetyl-D,L-2,6-dimethyltyrosine methyl ester in 25% aq. methanol, pH 7.90, 25.0°, 0.30 M NaCl, $[S]_o$ = 1.92×10^{-3} M $[E]_o$ = 0.21 mg. P.N./ml.

Table I

α-Chymotrypsin Catalysed Hydrolysis of N-Acetyl-D,L-2,6-dimethyltyrosine Methyl Ester at pH 7.90 and 25.0° C.

$(S)_o$ [a]	$(E)_o$ [b]	Solvent [c]	$(NaCl)$ [d]	v_o [e]
1.92	0.21	25% aq. methanol	0.30	6.8 [f]
"	---	"	"	1.5
2.10	---	20% aq. acetone	"	3.7
"	0.20	"	"	12.2 [f]
2.02	1.03	10% " "	0.20	146 [f]
"	1.07	15% " "	"	109 [f]
"	1.11	20% " "	"	81 [f]

a) in $M \cdot 10^{-3}$

b) in mg. P. N./ml.

c) V:V

d) in M

e) in $(M/min) \cdot 10^{-6}$

f) corrected for "blank" reactions

assigned the D configuration to the levorotatory form since it was isolated, as the p-toluenesulfonate, in the urine of a dog subcutaneously injected with the levorotatory acid. Furthermore, if the pure antipodes were treated with nitrosyl bromide to form optically active α-bromo-neocaproic acids and appropriate derivatives of these acids used to acylate L-tyrosine, only the derivative of d-t-leucine was hydrolysed by trypsin. The bromo acids were aminated to give amino acids of the same configuration as the original acids.

Greenstein (33), noting that Abderhalden's assignment violates the Lutz-Jirgensons rule (32, 34, 35), investigated the hydrolysis of the racemic amide mixture by a purified amidase from hog kidneys and found that the amide of the levorotatory acid was selectively hydrolysed. Greenstein pointed out that the transformations of several amino acids, including valine (36), to the corresponding bromo acids and the reverse transformation proceed by Walden inversion. Greenstein's rejection of Abderhalden's assignment of configuration is also supported by rotary dispersion studies (37). Greenstein's assignment, namely that the levorotatory acid has the L configuration, will be adopted in this thesis.

Resolution of N-acetyl-D,L-β,β-dimethylphenylalanine was attempted by fractional crystallization of the salts with ℓ-α-phenylethylamine in acetone, methanol, water, isopropanol and n-butanol, and of the salts with ℓ-2-aminobutanol-1 in n-butanol-benzene-ligroin mixture, acetone, dioxane, dimethylformamide, dimethylsulfoxide,

acetonitrile and in n-butanol-ethyl acetate mixture. In no case was a high degree of resolution obtained. However, small excesses of one antipode can be separated from N-acetyl-D,L-β,β-dimethylphenylalanine by fractional crystallization from water, the pure antipode having a greater water solubility. N-Acetyl-d-β,β-dimethylphenylalanine, $[\alpha]_D$ = + 42.7°, m.p. 157.5-158.0° , and N-acetyl-l-β,β-dimethylphenylalanine, $[\alpha]_D$ = - 44.6° , m.p. 158.5-159.0° were isolated, in 8% and 24% yields respectively, by fractional crystallization of the ℓ-α-phenylethylamine salts from acetone.

N-Acetyl-D,L-2,6-dimethyltyrosine methyl ester is hydrolysed stereospecifically by α-chymotrypsin. After hydrolysis of a solution of the D,L mixture, in 20% aqueous acetone, 0.20 M NaCl and pH 7.90 in the presence of 0.85 mg. P. N./ml. α-chymotrypsin, for 1.5 hours, levorotatory ester $[\alpha]_D$ = - 17.8°, was recovered in 28% yield. Due to the formation of large amounts of denatured enzyme during the recovery attempt, no acidic products were isolated. Resolution of O,N-diacetyl-D,L-2,6-dimethyltyrosine was unsuccessfully attempted by fractional crystallization of the salts with ℓ-2-aminobutanol-1 from acetone, methanol, water, dimethylformamide and acetonitrile.

Experimental:

4,6-Di-t-butylpyrogallol: A mixture of conc. H_2SO_4 (30 ml., 0.54 mole), pyrogallol (75.6 g., 0.60 mole), t-butanol (150 ml., 117 g., 1.58 mole) and glacial acetic acid (150 ml.) was stirred for five hours, after which time all of the pyrogallol had dissolved and a deep, cherry red solution had formed. The solution was allowed to stand for 40 hours and then poured, with stirring, into 3 ℓ. of water. A pink precipitate formed and was collected and recrystallized from benzene, with an unsuccessful attempt at decolorizing with Norite, to obtain short, pink rods (30 g., 0.126 mole, 21% yield), m.p. 119-121° (lit. 121°) (38).

Permanganate Oxidation of 4,6-Di-t-butylpyrogallol: 4,6-Di-t-butylpyrogallol (25 g., 0.105 mole) was dissolved in 360 ml. of 0.8 M NaOH. Solid $KMnO_4$ (18 g., 0.113 mole) was added, followed by 12 smaller portions (5 g. each, 0.377 mole total) at ten minute intervals. The temperature of the reaction mixture was kept below 50° by addition of ice. The reaction was allowed to continue overnight and the excess manganate reduced by addition of methanol. MnO_2 was removed by filtration and washed with hot, dilute NaOH. The filtrate was acidified and extracted three times with ether. The ether extract was extracted three times with saturated aqueous $NaHCO_3$, dried over Na_2SO_4 and evaporated to a neutral residue which was recrystallized from benzene-ligroin to give an unidentified product (0.9 g.), m.p. 172-175° . The bicarbonate extract was acidified with HCl and extracted

three times with ether. The ether extract was dried over Na_2SO_4 and evaporated to an oily residue (13.5 g.).

One half of the product obtained above was dissolved in 25 ml. of 2 N NaOH and hydroxylamine hydrochloride (3.5 g., 0.050 mole) and Na_2CO_3 (5.0 g., 0.050 mole) were added. The mixture was warmed overnight, 30 ml. of water added and the solution acidified with HCl. A small amount of precipitate was collected and recrystallized from benzene-ligroin (1:1) to obtain an unidentified product, m.p. 180.5-182.0° .

The other half was dissolved in 30 ml. of methanol and conc. H_2SO_4 (1.5 ml.) added and the mixture refluxed overnight. The reaction mixture was extracted with ether and the ether extract dried over Na_2SO_4 and evaporated to an oil. This oil decomposed during attempted fractional distillation in vacuo.

Pivaloyl Chloride: Pivalic acid (25 g., 0.245 mole) was refluxed with thionyl chloride (45 ml., 60 g., 0.445 mole) for two hours. Pivaloyl chloride (25.7 g. , 0.214 mole, 87% yield), b.p. 93-98° (Lit. 105-106°) (39) was obtained by distillation.

Pivalaldehyde: t-Butanol (38 ml., 29.6 g., 0.40 mole), freshly distilled over CaO, was added with stirring to a chilled solution of $LiAlH_4$ (4.75 g., 0.125 mole) in 250 ml. of tetrahydrofuran (dried over KOH and distilled over $LiAlH_4$). The resultant solution was added, over a period of 15 minutes, to a solution of pivaloyl chloride (17.3 g., 0.144 mole) in 250 ml. of dry tetrahydrofuran, cooled to -75°. The mixture

was allowed to warm slowly to room temperature and 200 ml. of 10% $NaHSO_3$ added. The solid precipitate was filtered and washed with water and ether. The filtrate was separated into two phases by addition of salt. The organic layer was washed twice with 10% $NaHSO_3$, the aqueous layers were combined and washed three times with ether. The precipitate was suspended in water and the pH of the suspension adjusted to 9 by addition of Na_2CO_3 and the solution warmed. The bisulfite extracts were similarly treated and all of the aqueous solutions combined and extracted with ether. A trace of hydroquinone was added to the ether extract and the extract dried over Na_2SO_4. A few ml. of xylene were added and the solution distilled through a 30 cm. Vigreux column to obtain pivalaldehyde (1.75 g., 0.020 mole), b.p. 73°. (Lit. 74-75°) (40). The pressure bisulfite layers were treated with more Na_2CO_3 and distilled to give additional product (0.65 g., 0.0075 mole, 22% yield).

t-Leucine by the Strecker Synthesis: A mixture of pivalaldehyde (2.25 g., 0.026 mole) in 1 ml. of ether and a solution of NH_4Cl (1.55 g., 0.029 mole) in 5 ml. of water and NaCN (1.27 g., 0.024 mole) in 3.5 ml. of water was shaken in a tightly stoppered centrifuge bottle for 4 hours. The reaction mixture was transferred to a 100 ml. round bottom flask and acidified with 6.5 ml. of 12 N HCl. The mixture was distilled over a free flame until salt formed in the flask. The contents of the flask were washed into a crystallizing dish and evaporated to dryness. The residue gave a positive test for chloride ion with $AgNO_3$ and

a negative ninhydrin test.

Pinacolone: A mixture of pinacol hydrate (83 g., 0.340 mole) and 215 ml. of 6 N H_2SO_4 was distilled until organic material ceased to distill. The two phase distillate was separated and the organic layer dried over $CaCl_2$ and distilled. Pinacolone (28.7 g., 0.287 mole, 85% yield), b.p. 100-102° (Lit. 103-107°) (41) was obtained.

Trimethylpyruvic Acid: Pinacolone (14.5 g., 0.20 mole) was added to a solution of $KMnO_4$ (63 g., 0.39 mole) and NaOH (20 g., 0.50 mole) in 2 ℓ. of water. The mixture immediately became warm, changed color from purple to green and MnO_2 precipitated. The mixture was shaken in a tightly stoppered, thick walled bottle for 2 hours, after which the supernate appeared colorless. A small amount of Na_2SO_3 was added to reduce any residual amount of manganate and the mixture filtered through Celite. The MnO_2 precipitate was washed with warm dilute NaOH and the filtrate evaporated to dryness. The crystalline residue was dissolved in 300 ml. of water and acidified with conc. HCl. The mixture was extracted with five 50 ml. portions of ether and the ether extracts dried over Na_2SO_4 and evaporated to a pale yellow liquid. This liquid was distilled, in vacuo, to obtain trimethylpyruvic acid (14.9 g., 0.115 mole, 57% yield), b.p. $_{4mm}$ 51-52° (Lit. b.p.$_{10\ mm}$ 73.5-75.0°) (42).

2-Oximino-3,3-dimethylbutyric Acid: Trimethylpyruvic acid (14.7 g., 0.113 mole) was dissolved in a solution of anhydrous K_2CO_3 (13.9 g., 0.10 mole) in 40 ml. of water. Hydroxylamine hydrochloride

(11.7 g., 0.17 mole) was added and the mixture allowed to stand at room temperature for 30 hours, after which some large, colorless crystals had formed in the reaction mixture. The mixture was acidified with conc. HCl, whereupon a large amount of colorless, crystalline material precipitated. The crystals were collected and dissolved in ether. The filtrate was extracted five times with ether and the combined ether solutions dried over Na_2SO_4 and evaporated in vacuo at room temperature to dryness. If, as in other experiments, the ether was evaporated on a steam bath, a non-crystallizable oil, exhibiting an intense nitrile absorption in the infrared region, was obtained. The residue was dissolved in benzene and the solution evaporated in vacuo to white crystals, m.p. 81-85° (Lit. for monohydrate 85°) (43) which were collected and washed with hexane. Analysis indicated that the material was a 2:1 mixture of anhydrous and monohydrated forms of α-oximinoneocaproic acid (12.9 g., 0.087 mole, 77% yield).

Analysis:	Calculated for $C_6H_{11}NO_3$ (145):	C: 49.64%; H: 7.68%; N: 9.65%
	$C_6H_{13}NO_4$ (163):	C: 44.16%; H: 8.03%; N: 8.58%
	Found:	C: 47.68%; H: 8.08%; N: 9.27%

Recrystallization of a small sample of this material from benzene-hexane gave anhydrous material as long, white needles, m.p. 115-117° (Lit. 116-117°) (42).

D,L-t-Leucine: I. (By catalytic hydrogenation): 2-Oximino-3,3-dimethylbutyric acid (0.86 g., 0.0057 mole) was dissolved in 20 ml. of 95% ethanol and Pd-C catalyst (0.5 g.), $PdCl_2$ (0.08 g.) and conc. HCl (1.5 ml.) added. The mixture was shaken at room temperature under 20 atmospheres of hydrogen for 24 hours. The catalyst was removed by filtration and washed with 95% ethanol. The filtrate was evaporated to a white, crystalline residue. This residue was dissolved in the minimum amount of water, neutralized to pH 6 with NH_4OH and an equal volume of 95% ethanol added. No precipitate formed and the solution gave a negative ninhydrin test. The solution was evaporated to an ether soluble residue. A CCl_4 solution of this residue absorbed strongly in the infrared region at 2930, 2830, 1260 and 1117 cm^{-1} and moderately at 3300, 1735, 1707, 1442, 1379, 1349, 1042, 1020 and 862 cm^{-1}.

II. (By reduction with 2% Na-Hg): 2-Oximino-3,3-dimethylbutyric acid (3.8 g., 0.025 mole) was added to a mixture of 2% Na-Hg (190 g.) in 100 ml. absolute ethanol, maintained at 50-60°. The mixture was kept acid by addition of ca. 7 N ethanolic HCl (ca. 25 ml. added) using strips of filter paper, soaked in bromocresol green, as the indicator. After uptake of acid had ceased, an additional 100 ml. of ethanolic HCl was added. The supernatant solution was decanted and white solid that had formed on the mercury surface was dissolved

in water. The combined solutions were filtered and the filtrate evaporated on the steam bath until crystallization occurred. The solid that formed was redissolved by addition of water and the solution, while warm, saturated with H_2S. The mixture was filtered while warm and neutralized to pH 6.5 by addition of 10% Na_2CO_3. The solution was evaporated and the crystalline residue was continuously extracted with 350 ml. of absolute ethanol for 60 hours, after which the ethanolic extract gave a positive ninhydrin test. After the ethanolic solution was cooled for a week, D,L-t-leucine (0.25 g., 0.0019 mole, 8% yield), sublimes 195-270°, m.p. 270-275° (decomp.) (Lit.: sublime 250°) (19) was collected. No additional salt free product could be obtained.

III. (By $NaBH_4$ reduction): $NaBH_4$ (0.40 g., 0.010 mole) was added to a stirred solution of 2-oximino-3,3-dimethylbutyric acid (1.5 g., 0.010 mole) in 100 ml. of 1N $NaHCO_3$. An evolution of gas was observed. After gas evolution had ceased, the mixture was acidified to pH 4 with HCl and evaporated to dryness. The residue was dissolved in the minimum amount of water, neutralized to pH 7 by addition of 10% Na_2CO_3 and evaporated to dryness. Neither the residue nor its methanolic extract gave a positive ninhydrin test.

IV. (By Al-Hg reduction): 2-Oximino-3,3-dimethylbutyric acid (5.0 g., 0.033 mole) was dissolved in 100 ml. of 50% aqueous ethanol and the solution added to 2% Al-Hg (200 g.) and the mixture maintained at 50° for 24 hours, after which a gelatinous precipitate had formed in

the supernate. The mixture was acidified with HCl and the supernate decanted. The supernate was warmed on the steam bath, saturated with H_2S and filtered. The filtrate was neutralized to pH 6 with 10% Na_2CO_3 and evaporated to a gelatinous residue. The residue was continuously extracted with water for 6 hours. Large amounts of gelatinous material passed through the extraction filter. The extract was evaporated and the residue continuously extracted with methanol for 12 hours. As before, precipitate passed through the filter. The extract was filtered while warm through very retentive filter paper and the precipitate washed with water. The filtrate gave a positive ninhydrin test and was evaporated to a solid residue (3.15 g.) which underwent partial sublimation in the range 200-270° and decomposed slightly at 280°. Further isolation of the amino acid was not attempted.

V. (By Zn-acetic acid reduction): 2-Oximino-3,3-dimethylbutyric acid (5.0 g., 0.033 mole) and Zn dust (5.0 g., 0.077 mole) were refluxed in 250 ml. of 50% acetic acid for 40 hours, after which all of the Zn had dissolved. The solution was saturated with H_2S and the white precipitate removed by filtration. The filtrate was evaporated to a white solid. The solid was dissolved in 150 ml. of warm water and an insoluble residue removed by filtration. The filtrate gave a positive ninhydrin test and no precipitate with $AgNO_3$. The filtrate was evaporated to 20 ml. and, while warm, 150 ml. of acetone added. A colorless precipitate formed. The mixture was warmed and water added dropwise

until the precipitate had dissolved. After the solution cooled, D,L-t-leucine (1.92 g., 0.015 mole), subliming above 200°, m.p. 275-300° (decomp.), crystallized. Additional product (0.60 g., 0.005 mole, 60% total yield) was obtained by evaporating the mother liquor to dryness and recrystallizing the residue from aqueous acetone. The crude material, which contained salt impurities, was recrystallized from aqueous acetone to obtain large, irregular, hexagonal platelets, subliming above 200°, m.p. 275-280° (decomp.).

Analysis: Calculated for $C_6H_{13}NO_2$(131): C: 54.93%; H: 9.99%; N: 10.68%

Found: C: 55.28%; H: 9.99%; N: 10.71%

N-Acetyl-D,L-leucine: D,L-t-Leucine (0.50 g., 0.0038 mole) was dissolved in 5 ml. of 2N NaOH and the solution cooled. Acetic anhydride (0.5 ml., 0.54 g., 0.0053 mole) was added and the mixture shaken vigorously for 2 minutes and then carefully acidified by dropwise addition of conc. HCl. The precipitate that formed was collected and recrystallized from water to obtain N-acetyl-D,L-t-leucine (0.35 g., 0.0020 mole, 53% yield), m.p. 227-234° (decomp.).

Analysis: Calculated for $C_8H_{15}NO_3$(173): C: 55.47%; H: 8.73%; N: 8.09%

Found: C: 55.31%; H: 8.55%; N: 8.14%

N-Acetyl-D,L-t-leucine Methyl Ester: Crude N-acetyl-D,L-t-leucine (0.75 g., 0.00434 mole) was dissolved in a cold solution of thionyl chloride (0.5 ml., 0.83 g., 0.0061 mole) in 4.0 ml. of anhydrous

methanol. The mixture was allowed to warm to room temperature and stand overnight. The solution was evaporated to a clear oil which crystallized immediately when scratched. The solid was recrystallized from water to obtain N-acetyl-D,L-t-leucine methyl ester (0.31 g., 0.00166 mole) as thick prisms, m.p. 110-111°. Additional product (0.18 g., 0.00096 mole, 60% total yield) was obtained by evaporation of the mother liquor and recrystallization of the residue from water. Some product was apparently lost due to sublimation during evaporation of the mother liquor on the steam bath.

Analysis: Calculated for $C_9H_{17}NO_3$ (187): C: 57.73%; H: 9.15%; N: 7.48%

Found: C: 57.34%; H: 9.02%; N: 7.41%

N-Formyl-D,L-t-leucine: D,L-t-Leucine (2.1 g., 0.016 mole) was refluxed with formic acid (5.0 ml., 6.1 g., 0.13 mole) for 6 hours. When the reaction mixture was cooled, a colorless precipitate appeared. This precipitate was collected and washed with formic acid to obtain N-formyl-D,L-t-leucine (1.70 g., 0.0105 mole, 65% yield), m.p. 208-212°, (Lit. 210°) (32).

Resolution of N-Formyl-D,L-t-leucine: N-Formyl-D,L-t-leucine (1.60 g.) was dissolved in 40 ml. of warm, absolute ethanol and the solution added to a warm solution of brucine (4.00 g.) in 80 ml. of absolute ethanol. No crystallization occurred after the solution was cooled for 72 hours and seeded with a crystal of N-formyl-D,L-t-leucine. Evaporation of the solution to half its original volume and cooling for

24 hours failed to induce crystallization. The solution was evaporated to a slowly crystallizing oil. This material was dissolved in 100 ml. of warm absolute ethanol by refluxing the mixture for several hours. The solution was seeded with a small amount of the residual solid and cooled. After 36 hours, the crystalline precipitate that formed was collected and washed with ethanol to obtain the brucine salt of N-formyl-D-t-leucine (2.58 g.), m.p. 190-192° (Lit. 195°) (32). This material was dissolved in 30 ml. of water and 6 ml. of 1N NaOH added to the solution. The milky suspension was extracted four times with $CHCl_3$ and the aqueous phase was warmed on the steam bath to remove trace amounts of $CHCl_3$ and acidified with HCl. After the solution had cooled, N-formyl-D-t-leucine (0.50 g.), white crystals, m.p. 222-223° , $[\alpha]_D$ of Na salt = + 31.7° (Lit. 31.8°) (32), was obtained. The mother liquor of the brucine salt was evaporated to dryness and the residue treated exactly as the first fraction of the brucine salt to obtain impure N-formyl-L-t-leucine (0.49 g.), tan crystals, m.p. 219.5-220.5° , $[\alpha]_D$ of Na salt = -28.2° (Lit. -32.0°) (32). The mother liquor of this material was evaporated to dryness and the residue recrystallized from water, decolorizing with Norite, to obtain more impure L-compound, white platelets (0.10 g.), $[\alpha]_D$ of Na salt = - 17.0°.

D-t-Leucine: N-Formyl-D-t-leucine (0.47 g., 0.0030 mole) was refluxed in 5 ml. of 10% HBr for 1.5 hours. The solution was evaporated to dryness and the residue dissolved in the minimum amount

of water. The solution was neutralized to pH 7 with 1N NH_4OH, warmed, and 100 ml. of acetone added. After the solution was cooled overnight, D-t-leucine (0.25 g., 0.0019 mole, 63% yield) crystallized in long, serrated needles, subliming without decomposition 250-295° $[\alpha]_D$ = + 10.4° ((Lit. + 10.01°) (32), + 9.5° (33)). Evaporation of the mother liquor and recrystallization of the residue gave impure D-t-leucine (0.13 g.) $[\alpha]_D$ = + 8.4°.

L-t-Leucine: The impure N-formyl-D-t-leucine, described above (0.40 g., 0.0026 mole) was refluxed in 5 ml. of 10% HBr for 1.5 hours. The solution was evaporated to dryness and the residue dissolved in the minimum amount of water and decolorized with Norite. Acetone (150 ml.) was added and the warm solution neutralized to pH 7 by addition of 1N NH_4OH, whereupon a precipitate formed. The solution was warmed and water added dropwise until the precipitate dissolved. As the solution was cooled, impure L-t-leucine (0.20 g., 0.0015 mole, 58% yield) crystallized in fine needles, subliming without decomposition, 240-280° $[\alpha]_D$ = -8.6° (Lit. -10.15° (32), -9.7° (33)) Evaporation of the mother liquor and recrystallization of the residue gave white needles (0.07 g.), $[\alpha]_D$ = -7.2°.

α,α-Dimethylbenzyl Alcohol: To dry magnesium turnings (26 g., 1.07 mole) was added a small amount of a solution of bromobenzene (112 ml., 167.4 g., 1.07 mole) in anhydrous ether (500 ml.). Reaction was initiated by crushing a piece of magnesium with a glass rod. The rest of the bromobenzene solution was added at a rate sufficient to keep

the reaction mixture refluxing gently. The mixture was refluxed for 30 minutes after the completion of the addition of the bromobenzene. The mixture was cooled in an ice bath and anhydrous acetone (80 ml., 63.3 g., 1.09 mole) was added dropwise. The mixture was refluxed for 2.5 hours and cooled. Saturated aqueous NH_4OH (150 ml.) was carefully added and the single phase supernate decanted from the resultant granular precipitate. The precipitate was washed with ether and the combined ether solutions dried over Na_2SO_4 and evaporated on the steam bath to a high boiling liquid. This liquid was distilled in vacuo to obtain α,α-dimethylbenzyl alcohol (109 g., 0.80 mole, 75% yield), b.p.$_{4mm}$ 72-78° (Lit. b.p.$_{9mm}$ 91°) (44).

4,6-Di(α,α-dimethylbenzyl)pyrogallol: Pyrogallol (38.4 g., 0.30 mole) was dissolved in 100 ml. of glacial acetic acid containing 3 ml. of conc. H_2SO_4. This solution was added with stirring to a cold solution of α,α-dimethylbenzyl alcohol (100 g., 0.73 mole) in 100 ml. of glacial acetic acid. The orange solution was allowed to stand overnight and then poured into 700 ml. of water. The precipitate that formed was collected, dried and recrystallized from hexane to obtain 4,6-di(α,α-dimethylbenzyl)pyrogallol (90 g., 0.25 mole, 83% yield), m.p. 121-123° (Lit. 120-121°) (20). The use of α-methylstyrene, instead of α,α-dimethylbenzyl alcohol, in the same molecular proportions did not affect the yield or the procedure.

Permanganate Oxidation of 4,6-Di(α,α-dimethylbenzyl)-pyrogallol: 4,6-Di(α,α-dimethyl)benzylpyrogallol (28 g., 0.077 mole) was added to 1 ℓ. of 0.75N NaOH. The solid became pink but did not dissolve

appreciably, although the supernate had an orange color. The mixture was neutralized and the precipitate collected and dissolved in 1.7 ℓ. of 30% aqueous acetone. $KMnO_4$ (50 g., 0.32 mole) was added and the solution stirred overnight, after which all of the permanganate color had disappeared. NaOH (20 g., 0.5 mole) was added and the solution stirred for one hour. MnO_2 was removed by filtration and washed with warm, dilute NaOH. The filtrate was acidified and extracted twice with ether. The ether extract was extracted twice with saturated aqueous $NaHCO_3$ and the aqueous extract acidified. The ether layer was dried over Na_2SO_4 and evaporated to a yellow oil. The acidified aqueous phase was extracted with ether and the ether dried over Na_2SO_4 and evaporated. The residue was refluxed overnight in a mixture of 40 ml. of 90% denatured ethanol and 1 ml. conc. H_2SO_4. The ethanol was evaporated and the residual oil poured into water, extracted into ether and the ether solution washed twice with aqueous $NaHCO_3$ and once with water. The ether was dried over Na_2SO_4 and evaporated. Distillation of the residue was attempted at a pot temperature of 170° and a pressure of 8 mm. The oil decomposed before any material distilled.

Air Oxidation of 4,6-Di(α,α-dimethylbenzyl)pyrogallol: 4,6-Di-(α,α-dimethylbenzyl)pyrogallol (30 g., 0.083 mole) was dissolved in 900 ml. of methanol containing 45 ml. of 7N NaOH. The solution rapidly turned a deep violet. Oxygen was bubbled through the solution for two hours, the solution rapidly turning cherry red and then yellow.

The solution was evaporated to ca. 300 ml. and 750 ml. of water added. The solution was acidified with conc. HCl and extracted with three 100 ml. portions of ether. The ether solution was extracted with aqueous bicarbonate and then with 5% NaOH. The bicarbonate extract was acidified and allowed to stand for several hours. The yellow crystal cake which formed was collected and dried. This material was washed with hot hexane, leaving undissolved 3,5-di(α,α-dimethylbenzyl)coumalic acid (V) (15 g., 0.040 mole), m. p. 147-148°. An additional crop of this material (1 g., 0.003 mole, 52% total yield) crystallized from the filtrate. Evaporation of the mother liquor gave an orange oil which could not be crystallized. The neutral fraction and the small amount of material soluble in NaOH were impure. In one experiment, an apparently pure compound (X), m.p. 47.5-49.5°, was isolated from the neutral fraction by recrystallization from aqueous methanol. If oxidation time was extended to 5 hours, the lactone of 2,4-di(α,α-dimethylbenzyl)-4-hydroxycrotonic acid (IX) precipitated from the reaction mixture and was recrystallized from aqueous acetone, m.p. 134-135°. Infrared analysis of the neutral fractions of shorter oxidations showed that this material was a major component of the neutral fraction.

Several air oxidations were inadvertently run in methanol containing lead salt impurities. A description of such an experiment is given:

4,6-Di(α,α-dimethylbenzyl)pyrogallol (50 g., 0.138 mole) was dissolved in 1.5 ℓ. of Pb contaminated methanol and 75 ml. of 7N NaOH.

Oxygen was bubbled through the solution for 4 hours, after which time the solution was still a deep cherry red and a pink precipitate had formed. This precipitate was insoluble in organic solvents and water. When treated with conc. HCl, it formed a yellow color, which was extracted by ether, and $PbCl_2$. More of this Pb salt precipitated when the reaction solution was allowed to stand overnight. The solution was filtered and the filtrate was evaporated to a yellow green solid. This residue was dissolved in 500 ml. of water and the solution acidified with conc. HCl. The orange oil that separated was extracted into ether and the ether solution was extracted with aqueous $NaHCO_3$. The green aqueous phase was acidified to precipitate an orange oil. The ether phase was dried over Na_2SO_4 and evaporated to a dark, partially crystalline residue. The orange, acidic oil was extracted into ether and the ether solution dried over Na_2SO_4 and evaporated to an orange oil. Recrystallization of this oil from benzene-hexane gave soft, colorless crystals of 3,5-di(α,α-dimethylbenzyl)-2-ketohexene-4-dioic acid (VI) (16 g., 0.0544 mole, 39% yield), m.p. 116.5-118.0° . Recrystallization of the neutral fraction gave an unidentified product (XI), m.p. 170-174°. In other experiments using Pb contaminated methanol, V, rather than VI, was isolated in low yield.

Identity of V: Compound V, m.p. 149.0-149.5°.

Analysis: Calculated for $C_{24}H_{24}O_4$ (376): C: 76.57%; H: 6.43%

Found: C: 76.58%; H: 6.51%

Molecular weight (Rast (45)): 382

Neutralization equivalent: 358; pK_a: 4.6

Saponification and neutralization equivalent: 167

Infrared absorption spectrum (in $CHCl_3$): The material showed a broad, strong carbonyl absorption at 1725 cm^{-1} which may include a shoulder at ca. 1700 cm^{-1}. An OH stretching absorption is present.

Ultraviolet absorption spectra: The ultraviolet spectra in methanol has, in addition to phenyl absorption, a strong band, λ_{max} 3030 Å, ϵ_{max} 8,650. In 5% NaOH, the band appears at 3050 Å and disappears over a twelve hour period at room temperature.

Functional tests: This compound forms no precipitate with 2,4-dinitrophenylhydrazine and gives no color with $FeCl_3$.

Oxidation (vide infra): Alkaline permanganate oxidation of V gives α-keto-β-phenylisovaleric acid (isolated as the oxime) in 50-60% yield (based on two molecules of product), small amounts of oxalic acid and compound IX.

Identity of VI: Compound V was refluxed in 15 ml. of 1N KOH for 30 minutes, the reaction mixture acidified and extracted with ether. The ether was dried over Na_2SO_4 and evaporated to an oil which was crystallized from hexane to obtain crystals, m.p. 116.5-118.0°. Admixture of this material with a sample of VI did not depress the melting point.

Analysis: Calculated for $C_{24}H_{26}O_5$ (394): C: 73.07%; H: 6.64%

Found: C: 73.39%; H: 6.98%

Identity of IX: This compound crystallized in long needles, m.p. 134-135° , from aqueous acetone.

Analysis: Calculated for $C_{23}H_{26}O_2$(334): C: 82.60%; H: 7.84%

$C_{22}H_{24}O_2$(320): C: 82.46%; H: 7.55%

Found: C: 82.57%; H: 7.72%

Infrared spectrum (in $CHCl_3$): The infrared spectrum shows a sharp, intense carbonyl absorption at 1755 cm^{-1}. No OH stretching absorption is present.

Ultraviolet spectrum (in CH_3OH): The ultraviolet spectrum exhibits only phenyl group absorption.

Nuclear magnetic resonance spectrum: The nuclear magnetic resonance spectrum of IX, in $CHCl_3$, is shown in figure 2. The methyl group peaks (a) are split at low sweep speeds.

Solubility: The material is insoluble in water, aqueous $NaHCO_3$, 5% aqueous NaOH and 85% H_3PO_4. It is soluble in ether and conc. H_2SO_4.

Functional tests: The material gives no precipitate with 2,4-dinitrophenylhydrazine, no color with $FeCl_3$ and does not decolorize solutions of Br_2 in CCl_4 or aqueous $KMnO_4$. When IX (ca. 30 mg.) was refluxed in a mixture of 0.2 ml. of 6N NaOH and 1 ml. of 0.5N $NH_2OH \cdot HCl$ in 95% ethanol for 5 hours and the mixture acidified, the solution gave no color with $FeCl_3$. However, the infrared spectrum of the product showed that the carbonyl absorption was shifted to 1705 cm^{-1}, and an alcoholic stretch had appeared at 3500 cm^{-1}. Partition of this

product between aqueous bicarbonate and ether did not change the infrared spectrum of either fraction.

Studies on X: This material crystallizes from aqueous methanol in soft needles, m.p. 47.5-49.5°.

Analysis: Calculated for $C_{21}H_{24}O_3$(324): C: 77.75%; H: 7.46%

$C_{22}H_{26}O_3$(338): C: 78.07%; H: 7.74%

Found: C: 77.62%; H: 7.66%

Molecular weight (Rast): 332

Infrared spectrum (in $CHCl_3$): The infrared spectrum exhibits two strong carbonyl peaks at 1730 and 1710 cm^{-1} but shows no OH absorption. Other strong absorptions occur at 1368 and 1170 cm^{-1}.

Solubility: The material is insoluble in water, aqueous $NaHCO_3$, and 5% aqueous NaOH. It is soluble in ether and reacts with conc. H_2SO_4.

Functional tests: X does not form a precipitate with 2,4-dinitrophenylhydrazine or a color with $FeCl_3$. It does reduce ammoniacal silver ion. If X is hydrolysed with 3N HCl, the hydrolysate also reduces silver ion and also gives a red-violet color with $FeCl_3$.

Studies on XI: This material crystallizes from benzene-hexane, m.p. 170-174°.

Analysis: Calculated for $C_{24}H_{24}O_3$(360): C: 79.97%; H: 6.71%

$C_{24}H_{26}O_3$(362): C: 79.53%; H: 7.23%

Found: C: 79.78%; H: 7.12%

This material absorbs strongly in the infrared range at 1675 cm^{-1} and shows an OH stretch absorption.

Permanganate Oxidation of 3,5-Di(α,α-dimethylbenzyl)coumalic Acid: 3,5-Di(α,α-dimethylbenzyl)coumalic acid (34.8 g., 0.093 mole) was dissolved in 1150 ml. of 1N NaOH and $KMnO_4$ (50 g., 0.316 mole) was added. After 48 hours the excess manganate was reduced by addition of solid Na_2SO_3 (6 g., 0.048 mole). A colorless, flocculent precipitate formed above the MnO_2 precipitate. The mixture was filtered and the precipitate washed with 300 ml. of hot, dilute NaOH. More colorless material postprecipitated in the filtrate. The filtrate was acidified and extracted three times with ether, the ether dried over Na_2SO_4 and evaporated to a partially crystalline residue. This residue only partially dissolved in 140 ml. of 5% NaOH. The mixture was filtered and the precipitate washed with dilute NaOH. The precipitate was recrystallized from 90% ethanol and proved to be compound IX. In a subsequent experiment, the MnO_2 precipitate was continuously extracted with ether to obtain IX in 5% total yield. Hydroxylamine hydrochloride (14 g., 0.20 mole), dissolved in the minimum amount of water, was added to the filtrate. A precipitate appeared but redissolved on the addition of Na_2CO_3 (20.5 g., 0.193 mole). The pH of the mixture was 8. The mixture was warmed on the steam bath for 2 hours, diluted with 250 ml. of water and acidified with HCl. A colorless oil precipitated and crystallized, when cooled and seeded, to give crude α-oximino-β-phenylisovaleric acid (21.65 g.), m.p. 98-110°. The material was recrystallized from benzene-hexane to give pure product (16.6 g., 0.080 mole), m.p. 119-121° (decomp.).

(Lit. 123.0-123.5°) (20). The mother liquor of the crude product was extracted three times with ether and the ether dried over Na_2SO_4 and evaporated to a slowly crystallizing oil, m.p. 108-115°. Recrystallization gave white platelets (5.8 g., 0.028 mole, 58% total yield), m.p. 120-122° .

Analysis: Calculated for $C_{11}H_{13}NO_3$(207): C: 63.75%; H: 6.32%; N: 6.76%

Found: C: 63.82%; H: 6.29%; N: 6.91%

In a similar experiment, a 25% yield of oxalic acid was isolated from the mother liquor of the recrystallization of the crude oximino acid. If VI was oxidized in the same way, identical results were obtained.

D,L-β,β-dimethylphenylalanine: I. (By $SnCl_2$ reduction): α-Oximino-β-phenylisovaleric acid (1.00 g., 0.0048 mole) was added to a mixture of $SnCl_2 \cdot 2H_2O$ (2.5 g., 0.011 mole) in 10 ml. of conc. HCl. The suspension was allowed to stand at room temperature for 12 hours, after which all of the oximino acid had dissolved. After another 12 hours, a pasty material precipitated. This material was extracted with ether. The aqueous phase was warmed on the steam bath to expell ether, cooled and neutralized to pH 6 with NH_4OH. The mixture was filtered, the filtrate giving a negative ninhydrin test. The precipitate gave both positive ninhydrin and $AgNO_3$ tests but no salt free amino acid could be isolated by washing the precipitate with warm methanol and addition of water to the methanolic washings. The ether layer was dried with Na_2SO_4 and evaporated to a two phase residue. One of these phases was soluble in $CHCl_3$ and the infrared absorption spectrum of this

solution showed that this material was unreacted starting material. The other substance was not investigated.

II. (By catalytic reduction): α-Oximino-β-phenylisovaleric acid (3.45 g., 0.0167 mole) was mixed with Pd-C catalyst (1.0 g.), $PdCl_2$ (0.16 g.), 35 ml. of 95% ethanol and 3.5 ml. of conc. HCl. The mixture was shaken under 15 atmospheres of hydrogen at room temperature for 3 hours. The catalyst was removed by filtration and washed with absolute ethanol. The filtrate was evaporated to a partially crystalline residue. This residue yielded crude starting material (1.85 g., 54% recovery) on recrystallization from water. Starting material was recovered in equal yield after reaction for 24 hours.

III. (By reduction with 2% Na-Hg): α-Oximino-β-phenylisovaleric acid (5.18 g., 0.025 mole) was added to a mixture of 2% Na-Hg (193 g.) in 75 ml. of absolute ethanol, maintained at 45-55° by means of a water bath. The reaction mixture was kept acid to bromocresol green indicator by addition of 7 N ethanolic HCl. Agitation of the mixture was minimized. After 1 hour, uptake of acid ceased (21 ml. of acid were added). The mixture was carefully acidified with dilute HCl, the solution decanted and the mercury washed with water. A colorless solid on the mercury surface dissolved during washing with water. The supernate was filtered and the filtrate evaporated until crystallization occurred. Sufficient warm water was added to redissolve the crystalline material. Some insoluble oil was observed. The warm solution was saturated with H_2S, boiled to coagulate the grey precipitate that formed, and filtered.

The filtrate was neutralized to pH 6.5 with Na_2CO_3 and cooled for 2 days. A colorless precipitate of D,L-β,β-dimethylphenylalanine (1.2 g., 0.0062 mole), m.p. 225-230° (decomp.), was collected. The mother liquor was evaporated to 50 ml. and cooled to obtain a second crop of amino acid (1.0 g., 0.0052 mole), m.p. 235-237° (decomp.). A third crop (0.2 g., 0.0010 mole), m.p. 225-228° (decomp.), was obtained by further reduction of volume of the mother liquor. The crude product (total yield 50%) was recrystallized from water, m.p.230-240° (decomp.). (Lit. 240°) (20).

Analysis: Calculated for $C_{11}H_{15}NO_2$(193): C: 68.37%; H: 7.75%; N: 7.22%

Found: C: 68.24%; H: 7.82%; N: 7.25%

In another experiment characterized by cessation of uptake of acid before the theoretical amount was absorbed, the supernate of the mercury, after evaporation of the ethanol and replacement with water, was extracted with ether to remove the previously observed oily material. During extraction, some amino acid precipitated and was collected by filtration. The ether solution was dried over Na_2SO_4 and evaporated to a 21% recovery of starting material and a small amount of nitrogen free product, XII, m.p. 184-186° . The amino acid was produced in 30% yield.

In a third experiment, compound XII (3.0 g., from 12.0 g. of α-oximino-β-isovaleric acid) precipitated as a solid when the ethanol in the mercury supernate was replaced by water.

Analysis: Calculated for $C_{22}H_{26}O_7$(402): C: 65.65%; H: 6.51%

Found: C: 65.50%; H: 6.40%

Material contains ash.

N-Acetyl-D,L-β,β-dimethylphenylalanine: D,L-β,β-dimethylphenylalanine (8.9 g., 0.0046 mole) was dissolved in 90 ml. of 2N NaOH and the solution cooled in a salt-ice bath. Acetic anhydride (9 ml., 9.7 g., 0.095 mole) was added and the mixture shaken vigorously. The clear solution that resulted was acidified to pH 2 with HCl. White crystals were collected and recrystallized from water to yield N-acetyl-D,L-β,β-dimethylphenylalanine (9.9 g., 0.042 mole, 92% yield), m.p. 183.5-185.0° (Lit. 178-179°) (20).

Analysis: Calculated for $C_{13}H_{17}NO_3$(235): C: 66.36%; H: 7.28%; N: 5.95%

Found: C: 66.21%; H: 7.19%; N: 5.90%

N-Acetyl-D,L-β,β-dimethylphenylalanine Methyl Ester: N-Acetyl-D,L-β,β-dimethylphenylalanine (0.5 g., 0.0021 mole) was added to a chilled solution of thionyl chloride (0.25 ml., 0.42 g., 0.0031 mole) in 2 ml. of absolute methanol. The solution was allowed to stand at room temperature for 36 hours and evaporated to a pale yellow oil (0.5 g.) which was recrystallized from water to give N-acetyl-D,L-β,β-dimethylphenylalanine methyl ester (0.47 g., 0.0019 mole, 88% yield), m.p. 80-82° .

Analysis: Calculated for $C_{14}H_{19}NO_3$(249): C: 67.44%; H: 7.76%; N: 5.66%

Found: C: 67.43%; H: 7.68%; N: 5.62%

Resolution of D,L-α-Phenylethylamine (46): D,L-α-Phenylethylamine (100 g., 0.826 mole), freshly distilled in a CO_2 atmosphere, was added dropwise to a warm solution of d-tartaric acid (125 g., 0.835 mole) in 1600 ml. of reagent grade methanol. The solution was cooled overnight and the precipitate, in the form of thick clusters of needles, was collected and washed with 350 ml. of cold methanol to obtain fraction #1 (114.5 g.). The mother liquor was evaporated to 600 ml. and cooled to obtain fraction #2 (39.0 g.) as colorless needles. The mother liquor was again evaporated to 200 ml. and cooled to obtain fraction #3 (63.5 g.). Fraction #1 was dissolved in 500 ml. of water and 250 ml. of 4N NaOH was added. The mixture was extracted with five 100 ml. portions of ether and the ether dried over Na_2SO_4 and evaporated. The residue was distilled in vacuo (44 g.), b.p. $9.5\,mm^{64-66°}$, $\alpha_D = -14.5°$. Fraction #2, treated in a similar way, gave amine (12.5 g.), b.p. $3.5\,mm^{48-53°}$, $\alpha_D = -3.6°$. Fraction #3 gave amine (19.2 g.), b.p. $3\,mm^{48°}$, $\alpha_D = +24.7°$. The amine from fraction #3 was dissolved in 145 ml. of 95% ethanol and a solution of 2.6 ml. of conc. H_2SO_4 in 390 ml. of 95% ethanol added with stirring. The mixture was cooled overnight, filtered and the precipitate of amine sulfate (12.0 g.) washed with 150 ml. of cold 95% ethanol. This material was treated with base, extracted with ether, the ether dried over Na_2SO_4, evaporated, and the residue distilled to obtain d-α-phenylethylamine (5.75 g.), b.p. $_{1mm}^{35°}$, $\alpha_D = +36.3°$ (Lit. +37.2) (46). Fraction #1 was resolved, as before, with d-tartaric

acid. The first fraction to precipitate gave ℓ-α-phenylethylamine (17.8 g.), b.p.$_{5mm}$ 58° , α_D = -37.6° (lit. -38.3°) (46).

Resolution of D,L-2-Aminobutanol-1 (47): D,L-2-Aminobutanol-1 (110.6 g., 1.25 mole) was added to a cold solution of d-tartaric acid (186 g., 1.24 mole) in 300 ml. of water and the solution cooled overnight. Fraction #1 (109 g.), silky, colorless needles, was collected and washed with absolute ethanol. The mother liquor was evaporated to 200 ml. and the viscous mixture seeded with a crystal of fraction #1 to obtain fraction #2 (150 g.), which was washed with absolute ethanol. Fraction #1 was dissolved in 50 ml. of warm water and 60 ml. of absolute ethanol was added. The mixture was cooled to obtain fraction #1a (90 g.), m.p. 96-99°, $[\alpha]_D$ = + 10.5° (Lit. + 10.5°) (47). Fraction #2 was recrystallized from the mother liquor of fraction #1a to obtain fraction #2a. (140 g.). Fraction #1a was dissolved in 250 ml. of water. Solid $Ca(OH)_2$ was added until the pH was constant at 9.4. The calcium tartarate precipitate was filtered and washed with 50 ml. of water. The filtrate and washings were distilled at 25 mm. pressure through a 45 cm. glass ring column, to remove most of the water. The residue was distilled at low pressure through a 15 cm. Vigreux column to obtain ℓ-2-aminobutanol-1 (10.8 g.), b.p.$_{1mm}$ 43° , $[\alpha]_D$ = - 9.4° (Lit. -9.9°) (47). Considerable decomposition occurred and a large, tarry residue remained after distillation.

Resolution of N-Acetyl-D,L-β,β-dimethylphenylalanine: I. N-Acetyl-D,L-β,β-dimethylphenylalanine (3.72 g., 0.0158 mole) was

dissolved in 200 ml. of warm acetone and ℓ-α-phenylethylamine (1.0 ml., 0.95 g., 0.0079 mole) added. Crystallization occurred within one minute. The mixture was cooled overnight to obtain amine salt (2.74 g.), as colorless needles, which was washed with 100 ml. of cold acetone. The filtrate was evaporated and recrystallized from water to obtain fraction #2 (1.10 g.), m.p. 183.5-185.0°, $[\alpha]_D$ = -0.8°. The salt precipitate was dissolved in 300 ml. of warm water and the solution acidified with HCl to obtain fraction #1 (1.15 g.), m.p. 184.0-184.5° . The mother liquor of fraction #1 was evaporated to 75 ml. and cooled. Only a small amount of crystals, fraction #1b (0.06 g.), m.p. 181-183°, formed. The mother liquor was neutralized to pH 8, extracted three times with ether and reacidified. This solution was evaporated nearly to dryness and the precipitate that formed collected and washed with water to obtain a salt contaminated material (0.89 g.). Recrystallization from water gave fraction #1c (0.14 g.) in long prisms, m.p. 157.5-158.0° , $[\alpha]_D$ = +42.7°. The mother liquor of fraction #2 was evaporated nearly to dryness. A colorless oil formed and slowly crystallized to a mixture of an amorphous powder (0.40 g.), m.p. 155-157°, resolidify and melt 183.5-185.0°, $[\alpha]_D$ = - 40.6°, and large rhombic crystals (0.03 g.), m.p. 158.5-159.0° , $[\alpha]_D$ = - 44.6°.

II. The D,L-acetyl amino acid (3.72 g.) was dissolved in 75 ml. of methanol and the solution warmed. ℓ-α-Phenylethylamine (1.0 ml., 0.95 g.) was added and the solution cooled. No crystallization occurred in 24 hours. The solution was evaporated to 35 ml. and cooled to obtain

amine salt (1.85 g.). The salt (fraction #1) was dissolved in 200 ml. of warm water and acidified with HCl to obtain crystals (0.60 g.), m.p. 184-185° , $[\alpha]_D = +1.4°$. The mother liquor was evaporated to 60 ml., made alkaline and extracted with ether. The aqueous phase was reacidified and extracted three times with ether, and the ether dried over Na_2SO_4 and evaporated to a clear oil which rapidly crystallized (0.22 g.), m.p. 157°, resolidified and melted 183.5-185.0° . The methanolic mother liquor of the salt was evaporated to 15 ml. and cooled. A mixture of hard, thick crystals and soft, white needles (1.63 g.) (fraction #2) was collected. Fraction #2 was dissolved in 150 ml. of water and acidified with HCl to obtain free acid (0.99 g.), m.p. 183.5-185.0° . The methanolic mother liquor was evaporated to dryness and the residue (fraction #3) was recrystallized from water to obtain crystals (0.58 g.), m.p. 182.0-184.5°.

III. The D,L-acetyl amino acid (1.86 g., 0.0079 mole) was dissolved in 100 ml. of acetone and ℓ-α-phenylethylamine (1.0 ml., 0.95 g., 0.0079 mole) added. The salt (2.70 g.) was collected and dissolved in 300 ml. of warm water. No crystallization occurred. The solution was evaporated in vacuo to 150 ml., whereupon crystals formed. The mixture was warmed to redissolve the crystals and cooled. Fraction #1 (1.70 g.) was collected, dissolved in 150 ml. of warm water, and acidified to obtain free acid (0.90 g.), m.p. 183-185° , $[\alpha]_D = -1.8°$. The filtrate of the salt (fraction #2) was warmed and acidified to obtain crystals

(0.44 g.), m.p. 183-185°.

IV. The D,L-acetyl amino acid (1.76 g., 0.0078 mole) was dissolved in 100 ml. of warm isopropanol and ℓ-α-phenylethylamine (0.5 ml., 0.48 g., 0.0039 mole) added. After the solution had stood for several hours, crystallization occurred. The mixture was stored in the refrigerator for 48 hours and fraction #1 (1.62 g.) was collected and washed with cold isopropanol. The filtrate was evaporated to fraction #2 (0.71 g.), an oil which solidified, m.p. 160-180°. Recrystallization from water gave fraction #2a (0.48 g.), m.p. 179-185°, $[\alpha]_D$ = - 3.0°. The mother liquor (2b) was evaporated and the residue recrystallized from water to obtain fraction #2c (0.05 g.), m.p. 182-184°. The mother liquor was evaporated to fraction #2d (0.15 g.), m.p. 130-180°, which, on recrystallization from water, gave fraction #2e (0.05 g.), m.p. 157.5-159.0°, $[\alpha]_D$ = - 36.0°. Fraction #1 was dissolved in water and acidified to obtain crystals (0.92 g.), m.p. 181-183°.

V. The D,L-acetyl amino acid (1.86 g.) was dissolved in 100 ml. of warm n-butanol and ℓ-α-phenylethylamine (0.5 ml.) added. As the solution was slowly cooled, fraction #1 of salt (1.53 g.) crystallized slowly and was collected. The mother liquor was evaporated to fraction #2 (0.93 g.), a slowly crystallizing oil, m.p. 155-170°. This material was recrystallized from water to give fraction #2a (0.52 g.), m.p. 182-184°. The mother liquor was evaporated to fraction #2b (0.30 g.), m.p. 145-190°. Recrystallization gave fraction #2c (0.06 g.), m.p. 180-184°. Evaporation of the mother liquor gave fraction #2d, m.p.

175-190° . The free acid from fraction #1 (0.78 g.), m.p. 182-185°, was obtained in the usual manner.

VI. The D,L-acetyl amino acid (1.47 g., 0.0063 mole) was dissolved in 5 ml. of warm n-butanol and ℓ-2-aminobutanol-1 (0.6 ml., 0.56 g., 0.0063 mole) was added, followed by 5 ml. of benzene and 5 ml. of ligroin (b.p. 80-100°). A flocculent precipitate formed immediately and could not be redissolved by heating. The mixture was cooled for 6 hours and the precipitate collected and washed with 15 ml. of a cold solution of equal amounts of n-butanol, benzene and ligroin to obtain fraction #1 (1.69 g.). The mother liquor was evaporated to a slowly crystallizing oil which was dissolved in 35 ml. of warm water and acidified. No crystallization occurred. Recrystallization of fraction #1 from 90% aqueous acetone was attempted but no material crystallized. The solvent was evaporated and recrystallization of the residue attempted from methanol-acetone (1:4). No crystallization occurred and the solvent was evaporated. The free D,L acid (1.12 g.) was isolated in the usual manner from the residue.

VII. The D,L acid (0.50 g., 0.00212 mole) was dissolved in 50 ml. of warm acetone and ℓ-2-aminobutanol-1 (0.1 ml., 0.093 g., 0.00105 mole) added. After 3 days crystallization had occurred. The precipitate (0.19 g.) was dissolved in 25 ml. of warm water and acidified with HCl. No crystallization occurred and evaporation of the solvent gave a non-crystallizable yellow oil. The mother liquor of the salt was evaporated and the residue recrystallized from water to obtain crystals (0.21 g.), m.p. 182-184° .

VIII. The D,L acid (1.00 g., 0.0043 mole) was dissolved in 50 ml. of warm, technical grade dioxane and ℓ-2-aminobutanol-1 (0.20 ml., 0.186 g., 0.0021 mole) was added. Crystallization occurred after 30 minutes of slow cooling. Fraction #1 (0.42 g.) was collected, dissolved in 30 ml. of warm water and acidified with HCl to obtain free acid (0.30 g.), m.p. 182-185° . The mother liquor was evaporated to a brown oil which crystallized on contact with warm water to pale orange crystals. Recrystallization from water gave fraction #2 (0.27 g.), pale orange crystals, m.p. 175-185°. The mother liquor was evaporated to a mixture of long white needles and a brown water soluble oil. The crystals (0.24 g.), m.p. 180-185°, were collected and washed with water.

IX. The D,L acid (1.00 g.) and ℓ-2-aminobutanol-1 (0.20 ml.) were mixed in 50 ml. of dimethylformamide in the usual way, Fraction #1 (0.11 g.) crystallized and was dissolved in 5 ml. of water and acidified. A small amount of material crystallized but was not investigated further. The filtrate was evaporated to a mixture of crystals and oil which was crystallized from water to obtain fraction #2 (0.20 g.), orange crystals, m.p. 178-184°. The mother liquor was evaporated to dryness and the residue collected and washed well with water to obtain fraction #3 (0.18 g.), pale orange crystals, m.p. 182-184°. The filtrate was acidified with HCl to obtain fraction #4 (0.16 g.), m.p. 182.5-184.0°.

X. The D,L acid (1.00 g.) and ℓ-2-aminobutanol-1 (0.20 ml.) was mixed in the usual manner in 50 ml. of dimethylsulfoxide. No

crystallization occurred. The solvent was evaporated and the residue recrystallized from acetonitrile. Amine salt (0.52 g.) crystallized and yielded free acid (0.35 g.), m.p. 181-185°. The mother liquor was evaporated and the residue recrystallized from water to obtain crystals (0.43 g.), m.p. 182.0-184.5°.

XI. The D,L acid (1.00 g.) and ℓ-2-aminobutanol-1 (0.20 ml.) were mixed in 80 ml. of a warm solution of ethyl acetate and n-butanol (5:3). Amine salt (0.56 g.) crystallized and yielded free acid (0.31 g.), m.p. 184-186°. The mother liquor was evaporated and the residue recrystallized from water to obtain crystals (0.38 g.), m.p. 182-185°.

O-Carbethoxy-3,5-dimethylphenol: 3,5-Dimethylphenol (97.6 g., 0.80 mole) was dissolved in 600 ml. of reagent grade benzene and pyridine (65 ml., 64 g., 0.81 mole) added. Ethyl chlorocarbonate (100 g., 0.92 mole) was added dropwise to the well stirred mixture. Pyridinium chloride was removed by filtration and the filtrate evaporated to an oily residue. The residue was distilled, in vacuo to obtain O-carbethoxy-3,5-dimethylphenol (113 g., 0.58 mole, 73% yield), b.p.$_{1.5mm}$ 90-91°.

Chloromethylation of O-Carbethoxy-3,5-dimethylphenol: I.: Crude, undistilled O-carbethoxy-3,5-dimethylphenol (30 g., 0.156 mole) was mixed with 37% aqueous formaldehyde (30 ml., 0.37 mole) and 125 ml. of conc. HCl. Anhydrous HCl was bubbled through the reaction mixture at 60-70° for 7 hours after which time a waxy solid had precipitated. Recrystallization of this precipitate from benzene-hexane gave soft, colorless crystals of 3,5-dimethyl-2,4,6-trichloromethylphenol (11.9 g., 0.045 mole). Additional crude product (3.7 g., 0.014

mole) was obtained from the mother liquor of recrystallization. The crude material (total yield 38%) was recrystallized twice to obtain soft, white crystals, m.p. 143-147°.

Analysis: Calculated for $C_{11}H_{13}OCl_3$ (267.5):

C: 49.38%; H: 4.81%; Cl: 39.75%

Found: C: 50.13%; H: 5.16%; Cl: 39.29%

II. O-Carbethoxy-3,5-dimethylphenol (113 g., 0.58 mole) was mixed with 37% aqueous formaldehyde (120 ml., 1.48 mole) and 570 ml. of conc. HCl. The mixture was kept at 50-60° and anhydrous HCl introduced for 3.5 hours. The solid that formed when the reaction mixture was cooled was extracted into $CHCl_3$. The $CHCl_3$ layer was dried over Na_2SO_4 and evaporated in vacuo. The residue was distilled in vacuo to to obtain O-carbethoxy-4-chloromethyl-3,5-dimethylphenol (98.5 g., 0.41 mole, 71% yield), b.p.$_{6mm}$ 169-170°. After this product had distilled, decomposition of the residue occurred, with evolution of HCl and the formation of a brittle polymer. The analytical sample was obtained from a similar experiment, b.p.$_{3mm}$ 145° (Lit. b.p.$_{3mm}$ 151-153°) (29).

Analysis: Calculated for $C_{12}H_{15}O_3Cl$ (242.5):

C: 59.38%; H: 6.23%; Cl: 14.61%

Found: C: 59.36%; H: 6.34%; Cl: 14.52%

The nuclear magnetic resonance spectrum (in CCl_4) of this material (fig. 3) shows that it is the symmetrical isomer.

Diethyl Acetamido(2,6-dimethyl-4-hydroxybenzyl)malonate:

Diethyl acetamidomalonate (13.0 g., 0.060 mole) was added to a sodium

ethoxide solution, prepared by adding freshly cut metal (1.4 g., 0.061 mole) to 200 ml. of absolute ethanol. After 10 minutes, O-carbethoxy-4-chloromethyl-3,5-dimethylphenol (15.2 g., 0.062 mole) was added to the cloudy solution and the mixture refluxed for 2 hours. The reaction mixture was cooled and the NaCl precipitate removed by filtration. The filtrate was evaporated in vacuo to a clear, viscous oil which formed soft, colorless crystals (12.2 g., 0.035 mole; 58% yield) when triturated with warm 20% aqueous acetone. The material was recrystallized from benzene, m.p. 152-154°, and from water 156-157°.

Analysis: Calculated for $C_{18}H_{25}NO_6$ (351): C: 61.52%; H: 7.17%; N: 3.99%

Found: C: 61.71%; H: 6.91%; N: 4.03%

D,L-2,6-Dimethyltyrosine: Crude diethyl acetamido(2,6-dimethyl-4-hydroxybenzyl)malonate (20.8 g., 0.059 mole) was refluxed in 90 ml. of 48% HBr under a nitrogen atmosphere for 3.5 hours. As soon as in vacuo evaporation of the reaction solution was attempted, crystallization occurred, indicating supersaturation of the solution. The mixture was cooled and orange crystals of D,L-2,6-dimethyltyrosine hydrobromide (16.1 g., 0.056 mole) were collected, washed with 50 ml. of 25% HBr and dried in vacuo. The material was slightly hydroscopic, gaining 0.8 g. in weight after overnight exposure to air. The hydrobromide was dissolved in 200 ml. of warm water and some insoluble material removed by filtration. The solution was neutralized to pH 6.5 with Na_2CO_3, whereupon the orange color disappeared. On the basic side of pH 6.5, the solution was violet and this color change was found to be a good

indicator for the neutralization. When the neutralized solution was cooled, crude D,L-2,6-dimethyltyrosine crystallized and was collected and washed free of colored material with acetone and then ether (5.1 g., 0.024 mole). Additional product (1.2 g., 0.006 mole) was obtained by partial reduction of the volume of the mother liquor. Recrystallization of the crude material (51% total yield) gave white, rhombic crystals, m.p. 230-231° (decomp.).

Analysis: Calculated for $C_{11}H_{15}NO_3$(209): C: 63.14%; H: 7.23%; N: 6.70%

Found: C: 62.94%; H: 7.07%; N: 6.51%

O,N-Diacetyl-D,L-2,6-dimethyltyrosine: Crude D,L-2,6-dimethyltyrosine (0.5 g., 0.0024 mole) was dissolved in 5 ml. of 2N NaOH, the solution cooled in a salt-ice bath and acetic anhydride (0.5 ml., 0.54 g., 0.0053 mole) added. The mixture was shaken and acidified with HCl to obtain an orange oil that slowly crystallized. The crude material was recrystallized from water, decolorizing with Norite, to obtain white crystals (0.40 g., 0.0013 mole, 54% yield), melting ca. 120°, resolidifying and melting sharply at 174-175°. After the material was dried at 60° in vacuo over P_2O_5, it melted sharply at 174-175°. Analysis suggests that this material is O,N-diacetyl-D,L-dimethyltyrosine monohydrate.

Analysis: Calculated for $C_{15}H_{21}NO_6$(311): C: 57.86%; H: 6.80%; N: 4.50%

Found: C: 57.69%, 57.72%; H: 6.71%, 6.80%; N: 4.33%

N-Acetyl-D,L-2,6-dimethyltyrosine Methyl Ester: O,N-Diacetyl-D,L-2,6-dimethyltyrosine monohydrate (0.5 g., 0.0016 mole)

was added to a chilled solution of thionyl chloride (0.25 ml., 0.42 g., 0.0031 mole) in 2 ml. of anhydrous methanol. The solution was allowed to warm to room temperature and stand for 36 hours. The solution was evaporated to a brown oil, which was triturated with water to form pale yellow crystals (0.33 g.), m.p. 190-193°. The crude material was recrystallized from water, decolorizing with Norite, to obtain white crystals of N-acetyl-D,L-2,6-dimethyltyrosine methyl ester (0.23 g., 0.00087 mole, 54% yield), m.p. 193.5-195.0°.

Analysis: Calculated for $C_{14}H_{19}NO_4$(265): C: 63.38%; H: 7.22%; N: 5.29%

Found: C: 63.45%; H: 7.16%; N: 5.28%

Resolution of O,N-Diacetyl-D,L-2,6-dimethyltyrosine: I. ℓ-2-Aminobutanol-1 (0.20 ml., 0.186 g., 0.0021 mole) was added to a warm solution of O,N-diacetyl-D,L-2,6-dimethyltyrosine monohydrate (1.10 g., 0.0035 mole) in 50 ml. of warm acetone. A gelatinous precipitate formed immediately. After 24 hours, the precipitate (0.48 g.) was collected, dissolved in 30 ml. water and acidified with 3 ml. of conc. HCl. No crystallization occurred. The solution was evaporated to dryness but the oily residue could not be crystallized. The mother liquor of the salt was evaporated to a yellow oil which crystallized on contact with warm water to white microcrystals (0.38 g.), melting ca. 120°, resolidifying and melting 173-175°, $[\alpha]_D = +1.5°$.

II. ℓ-2-Aminobutanol-1 (0.20 ml.) was added to a warm solution of D,L acid monohydrate (1.5 g., 0.0048 mole) in 25 ml. of dimethylformamide. No crystallization occurred and the solvent was evaporated.

Recrystallization of the residue from methanol was attempted but no crystallization occurred. The solvent was evaporated and the residue recrystallized from water to obtain pale yellow, optically inactive crystals (0.73 g.), m.p. 120-125°.

III. ℓ-2-Aminobutanol-1 (0.20 ml.) was added to a warm solution of D,L acid monohydrate (1.50 g.) in 100 ml. of acetonitrile. After three days, the amine salt (0.61 g.) was collected, dissolved in 15 ml. of H_2O and acidified to pH 4 by addition of 10 ml. of 0.2N HCl. No crystallization occurred. The mother liquor of the salt was evaporated to a yellow oil which crystallized from water to give optically inactive acid (0.53 g.).

Enzymatic Resolution of N-Acetyl-D,L-2,6-dimethyltyrosine Methyl Ester: A solution of α-chymotrypsin (1.15 g.) in 30 ml. of water, neutralized to pH 7.9, was added to a solution of N-acetyl-D,L-2,6-dimethyltyrosine methyl ester (0.253 g., 0.00095 mole) and NaCl (2.34 g., 0.04 mole) in 170 ml. of 23% aqueous acetone, neutralized to pH 7.9. The pH was maintained at 7.9 by addition of standard base from a burette. The original rate of production of acid was ca. 10^{-4} M/l min. The reaction was followed for 1.5 hours without a clear cessation of uptake of base (total base added: 0.900 millimoles). The reaction mixture was shaken with $CHCl_3$, causing large amounts of denatured protein to precipitate. The precipitate was removed by centrifugation and filtration through Celite and washed well with $CHCl_3$. The two phases of the filtrate were separated and the $CHCl_3$ layer evaporated

to a glass, m.p. 150-155°. Recrystallization of this material gave levorotatory N-acetyl-2,6-dimethyltyrosine methyl ester (35.4 g.), m.p. 156-158°, $[\alpha]_D = -17.8°$.

Analysis: (sample too small for precise results): C: 63.9%; H: 7.6%

The aqueous phase was acidified to pH 2, extracted with $CHCl_3$, denatured protein removed by filtration over Celite, and the $CHCl_3$ layer evaporated. A small amount of residue was left but it could not be recrystallized from water.

Methyl Pyruvate Phenylhydrazone: Pyruvic acid phenylhydrazone (8.1 g., 0.0455 mole) was dissolved in 300 ml. of cold anhydrous methanol and the solution saturated with anhydrous HCl. The mixture was left at room temperature overnight, evaporated to 100 ml. and 200 ml. of water added. The mixture was cooled and scratched to obtain crude methyl pyruvate phenylhydrazone (7.75 g., 0.0404 mole, 89% yield) as a pale yellow green solid, m.p. 70-80°.

Methyl Indole-2-carboxylate: Crude methyl pyruvate phenylhydrazone (7.4 g., 0.0385 mole) was mixed with polyphosphoric acid (13 g.) and the mixture warmed. At 70° an exothermic reaction occurred, the temperature rising to 130°. The mixture was maintained at 130° for 10 minutes, then cooled to 60° and 100 ml. of water added. The mixture was filtered and the yellow precipitate dried and recrystallized, decolorizing with Norite, to obtain nearly white needles (1.7 g., 0.0096 mole, 25% yield) of methyl indole-2-carboxylate, m.p. 149-151°. Recrystallization from benzene, decolorizing with Norite, gave white

needles (0.85 g.), m.p. 151-153° (Lit. 151-152°) (48).

Analysis: Calculated for $C_{10}H_9NO_2$(175): C: 68.56%; H: 5.18%; N: 8.00%

Found: C: 68.63%; H: 5.25%; N: 7.96%

Kinetic Procedure: The procedure, described in section I of this thesis, was used. For experiments at $(E)_o$ of 1 mg. P. N./ml., individual stock solutions, prepared just previous to the experiment, were used in order to avoid denaturation effects.

Discussion:

Any attempt to rationalize the dependence of rates of α-chymotrypsin catalysed hydrolyses of amino acid derivatives on the steric features of the side chain runs into the difficulty that, to a certain extent, groups γ, or further, from the carboxyl group exert an activating effect. The work of Jennings and Niemann (16), which showed that the kinetic constants for the α-chymotrypsin catalysed hydrolyses of acetyl-L-phenylalaninamide and acetyl-L-hexahydrophenylalaninamide are approximately the same, indicates that this effect is neither inductive nor due to primarily π electron binding of the aromatic ring. Furthermore, a comparison of the rates of enzymatic hydrolyses of N-acetyl-L-leucine methyl ester with those of amino acids with shorter side chains (table II), shows that γ aliphatic groups can have an activating effect similar to rings.

The inactivity of N-acetyl-D,L-t-leucine methyl ester and the results for the series leucine, isoleucine and valine (13) confirms the presence of a marked β-steric hindrance effect for aliphatic amino acids. Unfortunately, accurate kinetic constants have been obtained only for valine derivatives, N-acetyl-L-leucine methyl ester being hydrolysed too rapidly for accurate measurement by currently available means. It is not yet possible, therefore, to assign this effect to steric repulsions in the transition states or specific repulsions in the enzyme-substrate complexes.

In spite of the lack of kinetic constants, the inactivity of N-acetyl-D,L-β,β-dimethylphenylalanine methyl ester represents a useful result. The rate of enzymatic hydrolysis of this "substrate" is not only very much slower than the rate for N-acetyl-D,L-phenylalanine methyl ester but is slower than N-acetyl-D,L-valine methyl ester. While the addition of a β-phenyl group to alanine causes a marked increase in the susceptibility to enzymatic hydrolysis, addition of the same group to valine causes a large decrease in such susceptibility. If this results from a decrease in stability of the enzyme-substrate complex, i.e., an increase in K_s rather than a decrease in k_3, it could be due to the inability of the phenyl group to orient itself suitably for binding to the active site. This could be due to interference between the β-methyl groups and the o-hydrogens on the ring. However, if this results from a decrease in k_3, it may be similar to an effect, described by Newman (49), for acid catalysed esterification of aliphatic esters. In this case, substitution of groups more than two carbons from the carboxyl group exerts a noticeable rate suppressing effect in α and β branched compounds. This appears to be a result of compression, in this case in the transition state between the various branching substituents.

The decrease in the rates of enzymatic hydrolyses of 2,6-dimethyltyrosine relative to the corresponding tyrosine derivatives, offers a good measure of the magnitude of the effects described above. Molecular models show that the o-methyl groups in 2,6-dimethyltyrosine interact with the carboxyl groups to the same degree as the γ methyls

in leucine. The methyls should interact with the β methylene hydrogens to the same degree as the interaction between the β-methyls and o-hydrogens in β,β-dimethylphenylalanine. Thus, the rate suppression observed for 2,6-dimethyltyrosine is a result either of internal steric compression in the amino acid side chain or of specific interaction between groups on the substrate and the active site of the enzyme.

Table II

Rates of α-Chymotrypsin Catalysed Hydrolyses of Compounds of the Type $RCH(NHAc)CO_2Me$ at pH 7.90, 25°C and 0.02 M NaCl.

R	$(S)_o$ [a]	$(E)_o$ [b]	v_o [c]	K_s [d]	k_3 [e]	ref.
H-	20	0.16	1.0	30.7	0.195	48
L-CH_3-	23	0.15	11.1	611	18.8	48
L-$(CH_3)_2CH$-	21	0.15	4.4	125.6	2.33	13
L-$CH_3CH_2CH(CH_3)$-	21	0.15	5.9			"
L-$(CH_3)_2CHCH_2$-	18	0.0030	16			"

a) $M \cdot 10^{-3}$

b) mg. P. N./ml.

c) $(M/l) \cdot 10^{-5}$

d) $M \cdot 10^{-3}$

e) millimoles/mg. P. N. min.

Conclusion:

A study of the rates of α-chymotrypsin catalysed hydrolyses of the N-acetyl-D,L-methyl esters of several synthetic amino acids indicates the presence of the β-steric hindrance to α-chymotrypsin catalysed hydrolyses of amino acid derivatives. At present there are not sufficient data available to allow satisfactory elucidation of the exact nature of this effect.

Unlike some other "cyclic" amino acid derivatives, methyl indole-2-carboxylate is not a substrate of α-chymotrypsin.

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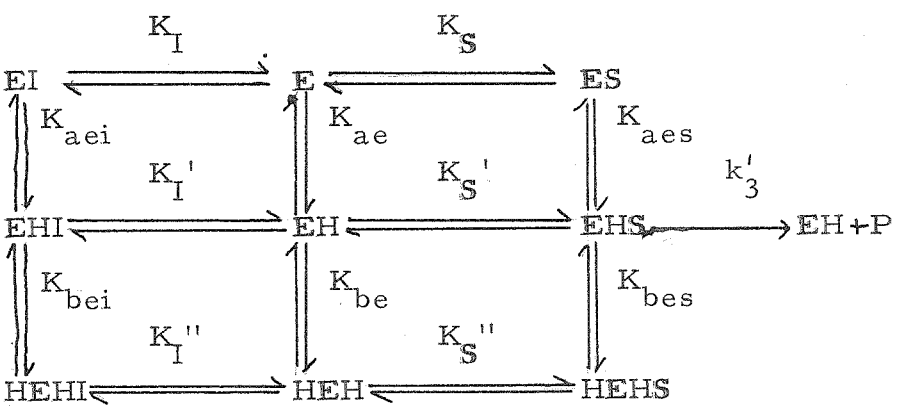
Propositions

I. Foster and Niemann (1) have found that while the K_I's toward α-chymotrypsin, of carboxylate inhibitors, decrease as pH is decreased from 7.9 to 6.9, the K_I of tryptammonium ion is constant over this range, rather than increasing due to a coulombic attraction. This apparent anomaly may be a result of the failure to consider the true microscopic enzyme-inhibitor dissocation constant. If protonation of a certain group in the enzyme tends to deactivate the enzyme, then the apparent K_I of an ammonium ion would not change over the specified range of pH.

Using an equilibrium system similar to that of Gordon (2):



$$v = \frac{k_3^{\circ}(S)_o(E)_o}{(S)_o + K_s^{\circ}(1 + (I)/K_I^{\circ})}$$

Where:

$$k_3^{\circ} = \frac{k_3'(E)_o(S)_o}{1 + K_{aes}/(H^+) + (H^+)/K_{bes}}$$

$$K_S^{\,o} = \frac{K_S'(1 + K_{ae}/(H^+) + (H^+)/K_{be})}{1 + K_{aes}/(H^+) + (H^+)/K_{bes}}$$

$$K_I^{\,o} = \frac{K_I'(1 + K_{ae}/(H^+) + (H^+)/K_{be}}{1 + K_{aei}(H^+) + (H^+)/K_{bei}}$$

If we use Gordon's values: $K_{be} = 10^{-6.9}$; $K_{ae} = 10^{-8.6}$ we can calculate the limiting values for the quantity:

$$R = (K_I^o \text{ at pH } 7.9)/(K_I^o \text{ at pH } 6.9)$$

As K_{bei} tends to 0, i.e., the inhibitor complexes only with the protonated form of the enzyme, R tends to 6.4. This describes the case for anionic inhibitors. If the inhibitor is neutral one would expect that K_{bei} would approximate K_{be} since Gordon found that neutral substrates did not change the dissociation constant of this group. In this case R approximates 1. For cationic inhibitors K_{bei} would tend to increase and R would approach 0.65.

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II. Nickon and Sing (1) have reported the reductive deamination of aliphatic amines by addition of hydroxylamine-O-sulfonic acid to an alkaline solution of the amine sulfonamide. The authors propose a mechanism involving elimination of sulfonic acid from the intermediate sulfon-1-alkylhydrazide to form the azo compound which then loses nitrogen to form the hydrocarbon. If this reaction can be extended to N-alkylhydroxylamine-O-sulfonic acids, dialkyl azo compounds could be generated under low energy conditions. This system would be particularly useful for the formation of unsymmetrical dialkyl azo compounds and an investigation of the tautomerism of these compounds in aqueous alkali to the two possible alkyl hydrazones.

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III. Budzckiewicz (1) reports the failure of 2-dichloromethyl-2-methylcyclohexadienone and 4-dichloromethyl-4-methylcyclohexadienone to rearrange to 2- and 4-dichloromethyl-m-cresol, respectively, in strong acid. This stability to acid may be a result of inductive destabilization by the chlorine atoms of the transition state for the 1,2-methyl shift. If this is true, a Hammett relationship should exist between the rates of pinacol rearrangements and the nature of substituents. The rates of rearrangements of 1,1,4,4-tetrasubstituted and

1,4-disubstituted pinacols appear to be a convenient system for study.

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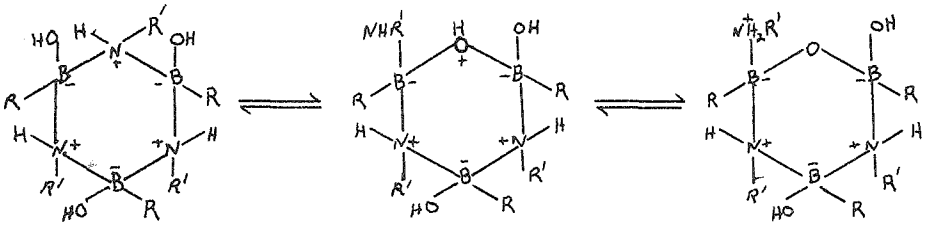
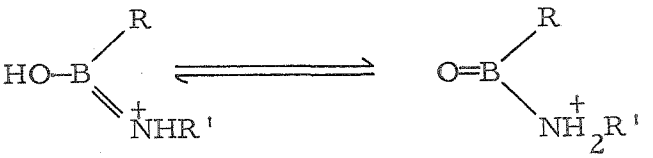
IV. The addition compounds of hexamethyl (1) and B-trimethylborazole (2) with water decompose to give trimethylboroxole while the addition compound of N-trimethylborazole (2) gives B-trihydroxy-N-trimethylborazole. This difference in behavior may indicate an equilibrium of the adduct between structures of the type:



I II III

Whether the decomposition to the boroxole is stepwise or, as proposed by Wilberg (1, 2), it proceeds by decomposition to the species:



might be determined by the decomposition products of the adduct of B-monomethyl-N-trimethylborazole. Stepwise reaction might form a stable boroxadiazole while breakdown according to the scheme of Wiberg

should give 33% trimethylboroxole and 66% B-trihydroxy-N-trimethyl-borazole.

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V. Chandross and Smolinsky (1) report the formation of 4,5,6-triphenyl-v-triazone on pyrolysis of 1-azido-1,2,3-triphenylpropane. They also report the formation of a compound, $C_{24}H_{30}N_2$, in 13% yield. This compound may be 2,3,4,6,7,8-hexaphenyl-1,5-diazocine, formed by dimerization and rearrangement of triphenylcyclopropane nitrine.

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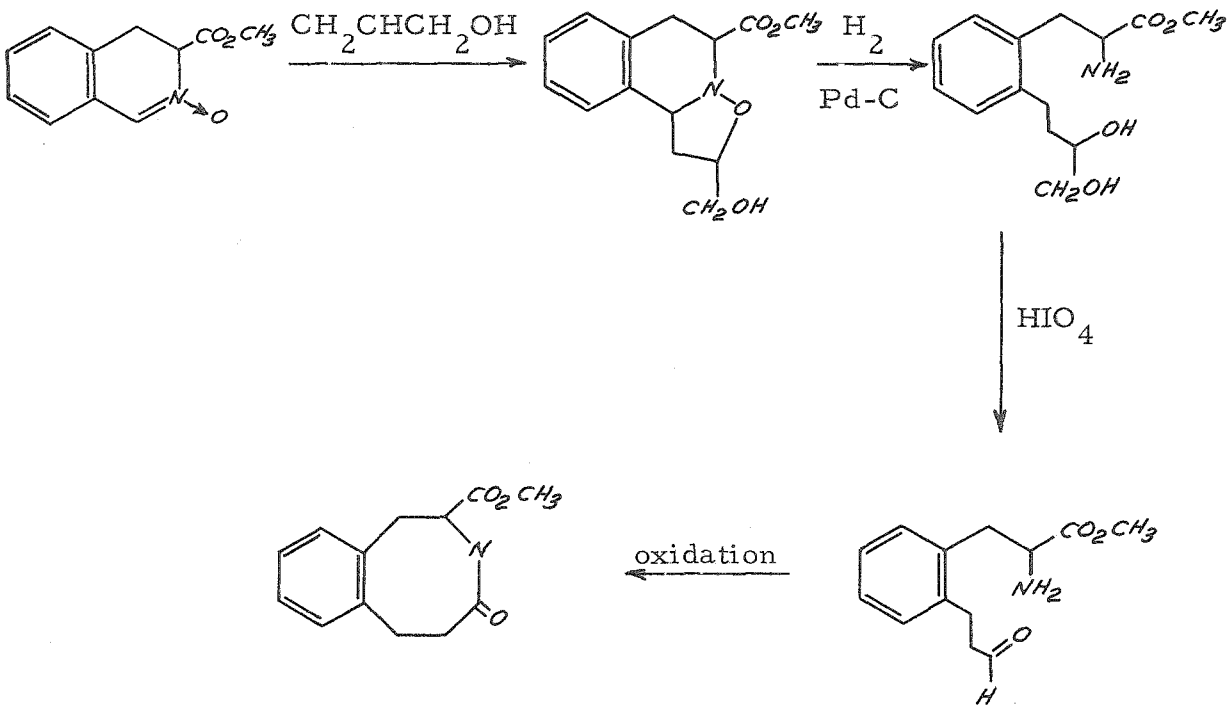
VI. Huisgen (1) has reported the ready addition of azomethine oxides to unsaturated functions and characterization of the isoxazolidine adduct by hydrogenation to give high yield N-O and O-N cleavage. Among the compounds that readily formed adducts was allyl alcohol. These reactions offer a useful method for two carbon ring expansion, especially for the synthesis of "bridged" amino acid homologues of 1-keto-1,2,3,4-tetrahydro-3-carbomethoxyisoquinoline.



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VII. Benkeser and Hickner (1) have found that the radical addition of siloform to various terminal alkines involves exclusive trans addition, to form the cis product. Skell attributes this result to the rigidity of the vinylic radical. This does not explain why the intermediate radical should form so that the aliphatic group and trichlorosilyl group are syn rather than anti to each other. It is proposed that the radical adduct has a bridged structure due to π orbital overlap with an available d-orbital of the Si. Geometric control is due to the steric blocking of the approach of a second siloform molecule from the same side as the trichlorosilyl group.

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VIII. Saffir and Taube (1) report that the rate of Cl_2 oxidation of oxalate present in the complex ion $(NH_3)_5CoC_2O_4^+$, falls off sharply after 80% conversion. This can be due to formation of the species $(NH_3)_5CoCO_2CO_2Co(NH_3)_5^{+4}$. Since the rates of oxidation of the monocobalt complex are much slower than the oxidation of free oxalate, the double complex might be expected to be even less active toward oxidation. The other oxidation that did not involve reduction of Co^{III}, the Mo^{VI} catalysed peroxide oxidation, did not show a falling off in rate. In this case the Mo may form a mixed complex and catalyse the oxidation by an insertion mechanism via a peroxymolybdic acid type species.

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