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Requirements for Stereospecificity in Hydrolysis by α-Chymotrypsin. III. The Acylamido Group[1]

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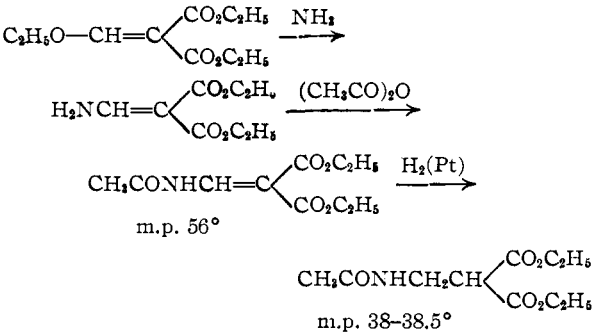
The compounds *dl*-ethyl-N-acetylalanine, $CH_3CH(NHCOCH_3)CO_2C_2H_5$ (I), *dl*-ethyl β-acetamidobutyrate, $CH_3CH(NHCOCH_3)CH_2CO_2C_2H_5$ (II), *dl*-ethyl β-phenyl-β-acetamidopropionate, $C_6H_5CH(NHCOCH_3)CH_2CO_2C_2H_5$ (III), diethyl α-acetamidomethylmalonate, $CH_3CONHCH_2CH(CO_2C_2H_5)_2$ (IV), and diethyl α-benzyl-α-acetamidomalonate, $CH_3CONHC(CO_2C_2H_5)_2CH_2C_6H_5$ (V), have been subjected to hydrolysis by α-chymotrypsin. Compounds I and III were hydrolyzed stereospecifically; compound IV was hydrolyzed with little or no stereospecificity; compounds II and V were not hydrolyzed by α-chymotrypsin. These results, combined with those previously reported for diethyl α-acetamidomalonate and diethyl β-acetamidoglutarate, indicate that an α- or β-acetamido group at a center or developing center of asymmetry is sufficient to lead to stereospecificity in hydrolysis of esters by α-chymotrypsin.

Our study of the hydrolysis of diethyl α-acetamidomalonate[2] and of diethyl β-acetamidoglutarate[3] has shown that these symmetric compounds are hydrolyzed slowly but asymmetrically by α-chymotrypsin and that the one active site of the enzyme, which is affected by the inhibitors hydrocinnamic acid and diisopropylphosphofluoridate, is catalytically active in these hydrolyses. The β-aryl substituent,[4] which is present in the typical natural substrates of this enzyme and in their competitive inhibitors, appears to be important for formation of the Michaelis complex and for high reactivity, and thus for chemical specificity, but is neither necessary nor sufficient[2] for stereospecificity. Interactions of the α- or β-acetamido group with the enzyme seemed, then, to be important for stereospecificity, and it is of interest to examine other substrates containing this substituent. We are now reporting on the action of α-chymotrypsin on the compounds: *dl*-ethyl-N-acetylalanine, $CH_3CH(NHCOCH_3)CO_2C_2H_5$, (I), *dl*-ethyl β-acetamidobutyrate, $CH_3CH(NHCOCH_3)CH_2CO_2C_2H_5$ (II), *dl*-ethyl β-phenyl-β-acetamidopropionate, $C_6H_5CH(NHCOCH_3)CH_2CO_2C_2H_5$ (III), diethyl α-acetamidomethylmalonate, $CH_3CONHCH_2CH(CO_2C_2H_5)_2$ (IV), and diethyl α-benzyl-α-acetamidomalonate, $CH_3CONHC(CO_2C_2H_5)_2CH_2C_6H_5$ (V).

Compounds I, II and III were prepared by conversion of the amino acids to their ester hydrochlorides, followed by acetylation. Compound III was also prepared by acetylation of the amino acid followed by esterification, but this sequence led to a lower yield. Compound IV was prepared by the following sequence of reactions: treatment of diethyl α-ethoxymethylenemalonate with aqueous ammonia, leading to diethyl α-aminomethylenemalonate, conversion of this to diethyl α-acetamidomethylenemalonate and hydrogenation to diethyl α-acetamidomethylmalonate. Hydrolyses were carried out at *p*H 7.8, standard alkali being added from an automatic buret controlled by a Radiometer Titrator. Readings were made of the volume of alkali consumed as a function of time, but variations in conditions necessary for kinetic analysis were not made at this time, the primary objective being to examine the stereospecificity of the reactions.



dl-Ethyl-N-acetylalanine (I) was hydrolyzed by α-chymotrypsin, the rate slowing down very markedly as the reaction approached 50% of completion, an indication that only one enantiomorph was reacting. One reaction was interrupted after 3.6 hr.; the solution was taken to dryness, and the unreacted ester was extracted and found to be optically active, $[\alpha]^{22}D$ +58°. A value reported[5] for L-ethyl-N-acetylalanine was $[\alpha]^{20}_{578}$ −66°, while that found for a sample synthesized in this work was $[\alpha]^{22}D$ −57°. From the residue, after extraction of the ester, the hydrolysis product L-N-acetylalanine was recovered in high yield, $[\alpha]^{22}D$ −59.7° (4.3% water), a value which compares well with those reported for this material, $[\alpha]^{16}D$ −45.6° (5% water),[6] $[\alpha]^{23}D$ −62° (3% water).[7] A second hydrolysis of the *dl*-ester was carried to 50% of completion in 3.5 hr. The unreacted D-ester was isolated, treated anew with α-chymotrypsin, and found to consume alkali very slowly. When corrections were applied for consumption of alkali by the enzyme and by non-enzymatic hydrolysis of the ester, the D-ester appeared to hydrolyze at a rate less than $1/_{200}$ of that of the L-ester. A third enzymatic hydrolysis of the *dl*-ester was allowed to proceed for 18 hr., essentially no alkali being consumed after 50% hydrolysis. The unreacted D-ester was isolated in 92% yield and characterized by its specific rotation, $[\alpha]^{22}D$ +64°, and infrared spectrum. The extended treatment with α-chymotrypsin appeared to lead to recovery of unhydrolyzed D-ester of high optical purity.

(1) We are pleased to acknowledge support of this work by the Division of Research Grants, The National Institutes of Health, RG 4584.

(2) S. G. Cohen and L. H. Klee, *J. Am. Chem. Soc.*, **82**, 6038 (1960).

(3) S. G. Cohen and E. Khedouri, *ibid.*, **83**, 1093 (1961).

(4) H. Neurath and G. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(5) K. Freudenberg and F. Rhino, *Ber.*, **57**, 1554 (1924).

(6) P. Karrer, E. Escher and R. Widmer, *Helv. Chim. Acta*, **9**, 34 (1926).

(7) M. L. Wolfrom, R. U. Lemieux and S. M. Olin, *J. Am. Chem. Soc.*, **71**, 2870 (1949).

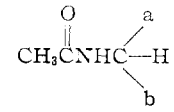
Unlike the proposed non-stereospecific hydrolysis of ethyl lactate,[8] the hydrolysis of *dl*-ethyl-N-acetylalanine by α-chymotrypsin proceeds stereospecifically, the α-acetamido group at a center of asymmetry being effective in the absence of a β-aryl group.

dl-Ethyl β-acetamidobutyrate (II), was not hydrolyzed by α-chymotrypsin although it was amply soluble in water, and the analog diethyl β-acetamidoglutarate (DEAG) was hydrolyzed slowly and asymmetrically.[3] When diethyl α-acetamidomalonate was added to the solution of *dl*-ethyl β-acetamidobutyrate and α-chymotrypsin, hydrolysis occurred readily, indicating that neither enantiomorph of the non-reacting butyrate was acting as an effective inhibitor. Considering the butyrate II and the glutarate DEAG as derivatives of β-alanine, the presence of a β-carboethoxymethyl substituent led to hydrolysis by α-chymotrypsin while a β-methyl did not.

The β-phenyl in *dl*-ethyl β-acetamido β-phenylpropionate (III) also led to hydrolysis by α-chymotrypsin. The material was only slightly soluble and was examined as a suspension, and hydrolysis was interrupted at only 22% of completion after 2 days. Nevertheless, optically active β-acetamido-β-phenylpropionic acid, $[\alpha]^{22}D$ −175° (1% ethanol), was obtained, and the recovered starting ester also showed optical activity. In this case we have introduced the β-phenyl group, the β-methyl leading to no observed hydrolysis, and it is difficult to evaluate the relative importance of the phenyl and acetamido groups to the stereospecificity. Association of a β-acetamido group with the enzyme may not leave the hydrolyzing group in close enough association with the active nucleophilic center of the enzyme unless a second large group—carboethoxymethyl or phenyl—is present to furnish either strong association or other steric interaction which leads to sufficient distortion of the active site and substrate to bring the groups that are to react into contact.

Diethyl α-acetamidomethylmalonate, $CH_3CONHCH_2$–$CH(CO_2C_2H_5)_2$ (IV), was hydrolyzed by α-chymotrypsin, but apparently not stereospecifically. In one experiment, carried to 87% reaction, the hydrolysate showed no optical activity. In a second, run at much higher concentration of substrate, there appeared to be some slight optical activity in the hydrolysate and in the isolated half-acid, but when the latter was converted to the 1,3-bis-(*p*-dimethylaminophenyl)-ureide, this was found to be inactive and identical with a synthetic racemic sample. In a third reaction, interrupted after 24% hydrolysis, no unequivocal evidence for optical activity due to the product was found. Compound IV is analogous to the malonate, which was hydrolyzed asymmetrically,[2] has its acetamido group beta to the ester groups like the glutarate,[3] and it showed reactivity to α-chymotrypsin similar to that of the glutarate.[3] Unlike both the previously studied glutarate and malonate esters, the acetamido group of compound IV is attached to a methylene group and not directly to a developing center of asymmetry, and this compound was not hydrolyzed with high asymmetry. Finally, α-benzyl-α-acetamidomalonate (V) was examined and found not to be hydrolyzed by α-chymotrypsin. Possessing both the β-aryl and α-acetamido groups of typical substrates, it has a quaternary α-carbon which prevents reaction.

Hydrolysis by α-chymotrypsin of substrates containing an α- or β-acetamido group at a center of asymmetry or a developing center of asymmetry proceeds stereospecifically. In the stereospecific hydrolysis of carboxylic esters containing the grouping



(1) if group a is carboethoxy, as in the α-acetamido compounds, group b may be a second carboethoxy, or methyl or benzyl and the symmetrical malonate requires no special conditions or explanation[2]; (2) if group a is carboethoxymethyl, as in the β-acetamido compounds, group b may be a second carboethoxymethyl or phenyl and these groups enhance the rate as compared with a methyl substituent which leads to no reaction; (3) neither a nor b may be a second hydrogen, a diastereomeric interaction at this carbon being important and not to be replaced by such an interaction at a more distant carbon.

Experimental[9]

α-Chymotrypsin was obtained from Worthington, salt free.

***dl*-Ethylacetylalanine.** — Dry hydrogen chloride was passed into a suspension of 36 g. (0.40 mole) of *dl*-alanine in 500 ml. of absolute ethanol until a clear solution resulted. This was boiled under reflux for 2 hr., evaporated in vacuum and dried, leading to a crystalline solid, ethylalanine hydrochloride,[10] 59.5 g. (0.38 mole), 95% yield. A portion of this (53.4 g., 0.34 mole) was heated on the steam-bath for 1.5 hr. with 71 g. of acetic anhydride and 86 g. of sodium acetate. Chloroform (100 ml.) was added, precipitated salts were filtered off and washed with chloroform. The solution was concentrated in vacuum and the product was distilled, 87° (< 1 mm.), 41.9 g. (0.26 mole), 77% yield, hygroscopic solid, m.p. *ca.* 35°, reported[11] b.p. 96° (1 mm.), reported[12] m.p. 38–39°.

Ethyl-L-acetylalanine was prepared, as described above, from 25 g. (0.26 mole) of L-alanine, 28 g. (0.18 mole), 68% yield, m.p. 34–35°, reported[5] 34–35°, α_{obsd} −8.02°, $[\alpha]^{22}D$ −57.3° (7% in ethanol). The L-alanine was from Nutritional Biochemicals Corp. α_{obsd} +0.59°, $[\alpha]^{22}D$ +9.8° (3.0% in *N* HCl), reported[7] +10° (3% *N* HCl).

Hydrolysis.—1. A solution of 1.024 g. (6.44 mmoles) of *dl*-ethylacetylalanine and 0.200 g. of α-chymotrypsin in 20 ml. of distilled water and 2 ml. of 0.1 *M* Na_2HPO_4 was allowed to react in the *p*H state at 25°, *p*H 7.85; 1.0 *N* sodium hydroxide was added automatically, 3.00 ml. (3.00 mmoles) being consumed in 219 min. The solution, volume 25 ml., was read in the polarimeter, α_{obsd} +1.01° (2 dcm.), α_{obsd} for chymotrypsin blank −0.48°. The solution was evaporated in vacuum at room temperature and the residue was extracted, first with ether and then with acetone, leading to 0.188 g. and 0.168 g., respectively, total yield 0.356 g. (2.24 mmoles), 65% yield, of the D-ester. The rotation was observed (a) 3.76% in chloroform, α_{obsd} −1.66°, $[\alpha]^{22}D$ −22.1°; (b) 6.75% in anhydrous ethanol, α_{obsd} +7.89°,

(8) I. Tinoco, *Arch. Biochem. and Biophys.*, **76**, 148 (1958).

(9) Elementary analyses are by Dr. S. M. Nagy, The Massachusetts Institute of Technology.

(10) S. D. Brewer and R. M. Herbst, *J. Org. Chem.*, **6**, 870 (1941).

(11) E. Cherbuliez and P. Plattner, *Helv. Chim. Acta*, **12**, 317 (1929).

(12) P. Karrer, E. Miyamichi, H. C. Storm and R. Widmer, *ibid.*, **8**, 208 (1925).

$[\alpha]^{22}D$ +58.4°. The residue from the extractions was dissolved in a few ml. of water, treated with 3.1 ml. of 1.0 *N* hydrochloric acid, concentrated to dryness in vacuum at room temperature and extracted with acetone. The extract was concentrated, leading to a residue, 0.528 g., which was washed with ether to remove residual ester, leaving L-acetylalanine, 0.358 g. (2.73 mmoles), 91% yield, m.p. 115–128°, reported[7] 112–128°, 122–123°, α_{obsd} −5.22°, $[\alpha]^{22}D$ −59.7° (4.3% in water).

2. A solution of 0.495 g. (3.10 mmoles) of *dl*-ethylacetylalanine and 0.200 g. of α-chymotrypsin in 20 ml. of water and 1.2 ml. of 0.1 *M* Na_2HPO_4 was allowed to react at *p*H 7.8; 1.535 ml. of *N* NaOH was consumed in 209 min., corresponding to 99% hydrolysis of one enantiomorph, at which point consumption of alkali had slowed down very markedly. Additional α-chymotrypsin (0.100 g.) was added, no effect on the hydrolysis resulting. The solution was taken to dryness in vacuum, the residue was extracted with five 30-ml. portions of acetone, and the acetone extracts were concentrated, leading to 0.180 g. (1.13 mmoles), 72% recovery, of the ethyl-D-acetylalanine, α_{obsd}, −1.69°, $[\alpha]^{22}D$ −22.1° (3.6% in chloroform). The D-ester was recovered from the chloroform, dissolved in 20 ml. of water and 2 ml. of phosphate buffer and treated with 0.100 g. of α-chymotrypsin, consuming 0.283 ml. of 0.1*N* NaOH in 60 min. An α-chymotrypsin blank consumed 0.189 ml. of 0.1 *N* NaOH; an ethylacetylalanine blank consumed 0.081 ml. in this period. Consumption of alkali due to enzymatic hydrolysis of D-ester appeared to be 0.013 ml. of 0.1 *N* NaOH in 60 min.

3. A solution of 2.000 g. (12.46 mmoles) of *dl*-ethylacetylalanine, 0.210 g. of α-chymotrypsin and 1.5 ml. of 0.1 *M* Na_2HPO_4 in 22 ml. of water was allowed to react for 18 hr. at *p*H 7.8, 6.31 ml. (6.31 mmoles) of 1 *N* NaOH being consumed. The solution was taken to dryness and extracted with acetone, leading to recovery of unreacted ethyl-D-acetylalanine, 0.917 g. (5.78 mmoles), 92%, α_{obsd} −1.81°, $[\alpha]^{22}D$ −23.6° (3.82% in chloroform), α_{obsd} +8.14°, $[\alpha]^{22}D$ +64° (6.36% in ethanol). The infrared spectrum was obtained in chloroform and was identical with that of the original *dl*-material: 2.91(w), 3.35(w), 5.75(s), 5.95(s), 6.62 (m), 6.87(m), 7.15(w), 7.24(m), 7.45(w), 7.64(w), 8.64(m), 9.80(m), 10.28(w), 11.65(w) μ.

DL-Ethyl β-Acetamidobutyrate.—Dry hydrogen chloride was passed into a suspension of 15 g. (0.145 mole) of β-aminobutyric acid (Nutritional Biochemicals Corp.) in 75 ml. of ethanol until a clear solution resulted. The solution was boiled under reflux for 0.5 hr., and concentrated in vacuum, leaving a viscous oil residue. This was boiled under reflux for 4 hr. with 15 g. of sodium acetate in 150 g. of acetic anhydride. Excess anhydride was taken off in vacuum, chloroform was added, salts were filtered off and the product was distilled, b.p. 115–116° (1 mm.), 14.3 g. (0.090 mole), 62% yield.

Anal. Calcd. for $C_8H_{15}NO_3$: C, 55.49; H, 8.67; N, 8.09. Found: C, 56.40; H, 8.63; N, 8.06.

A solution of 0.867 g. (5.01 mmoles) of *dl*-ethyl β-acetamidobutyrate, 0.8 ml. of 0.1 *M* Na_2HPO_4 buffer and 0.100 g. of α-chymotrypsin was allowed to react in the *p*H stat at *p*H 7.9, 22°, for 4 hr., 0.05 ml. of 1.0 *N* sodium hydroxide being consumed, an amount equal to that which would be consumed by the ester in the absence of enzyme. Some diethyl α-acetamidomalonate was added to the solution, rapid hydrolysis and consumption of alkali ensuing.

Ethyl β-Acetamido-β-phenylpropionate.—β-Amino-β-phenylpropionic acid was prepared in accordance with literature directions.[13] Malonic acid (20 g., 0.19 mole), benzaldehyde (20 g., 0.19 mole) and ammonium acetate (30 g., 0.39 mole) were heated on the steam-bath for 5 hr. in 40 ml. of ethanol. The product was collected, washed with alcohol and crystallized from water; 11.2 g. (0.068 mole), 36% yield, m.p. 216° dec., reported[13] 216°.

(1) A suspension of 18.8 g. (0.114 mole) of β-amino-β-phenylpropionic acid in 180 ml. of dry ethanol was saturated with dry hydrogen chloride at 0°, stirred for 4 hr., the hydrochloride dissolving, and refrigerated overnight. The solvent was removed in vacuum at room temperature, leaving the ester-hydrochloride, 25.8 g. (0.113 mole), 99% yield, m.p. 145–150°. A portion of this (9.18 g., 0.043 mole) was boiled under reflux for 6 hr. with 90 ml. of acetic anhydride and 3.5 g. (0.043 mole) of sodium acetate. The mixture was concentrated in vacuum and the residue was extracted with ether; the extract was washed with bicarbonate and concentrated, and the residue was chromatographed on alumina from ether and eluted with chloroform, leading to ethyl β-acetamido-β-phenylpropionate (6.5 g., 0.029 mole), 67% yield, in several fractions melting in the range 38–42°. It was recrystallized from hexane; m.p. 40.5–41.5°. The infrared spectrum was obtained on a Perkin–Elmer model 21 spectrometer in chloroform: 2.91(m), 3.00(w), 3.35(m), 4.08(w), 5.12(w), 5.31(w), 5.80(s), 5.97(s), 6.68(s), 6.90(m), 7.28(m), 8.50(m), 9.12(m), 9.70(m) μ.

Anal. Calcd. for $C_{13}H_{17}O_3N$: C, 66.38; H, 7.23; N, 5.96. Found: C, 67.08; H, 7.49; N, 6.21.

(2) β-Amino-β-phenylpropionic acid (20 g., 0.121 mole) was boiled under partial reflux with 220 ml. of freshly distilled acetic anhydride for 3 hr., 40 ml. of distillate being collected. The solution was concentrated in vacuum and the residue was stirred with 40 ml. of chloroform and 25 ml. of water, crystals slowly forming. This was washed with chloroform and water and crystallized from water, leading to **β-acetamido-β-phenylpropionic acid** (9.55 g., 0.046 mole), 38% yield, m.p. 175–176°, reported[14] 164°. The infrared spectrum was obtained in KBr: 2.99(m), 3.30(w), 3.45(w), 5.10(w), 5.85(s), 6.05(m), 6.15(s), 6.44(s), 6.67(w), 6.95(w), 7.03(w), 7.29(w), 7.46(w), 7.60(w), 7.90(m), 8.25(m), 8.50 (w), 9.22(w), 10.32(w), 10.68(w) μ.

Anal. Calcd. for $C_{11}H_{13}O_3N$: N, 6.76. Found: N, 6.78.

A portion of this compounds (3.0 g., 0.0145 mole) was converted to the ethyl ester (2.21 g., 9.4 mmoles), 65% yield, m.p. and mixed m.p. 39–41°.

Hydrolysis.—A suspension of 1.143 g. (4.86 mmoles) of *dl*-ethyl β-acetamido-β-phenylpropionate in 20 ml. of water was treated with 0.100 g. of α-chymotrypsin at *p*H 7.9, 24°, in the *p*H stat, 0.534 ml. of 1.0 *N* sodium hydroxide (0.53 mmole) being consumed in 51 hours, 22% reaction. The mixture was extracted with ether, treated with a stream of air to remove residual ether, shaken with 0.1 g. of charcoal, filtered and examined in the polarimeter, α_{obsd} −1.40°, corrected for rotation due to α-chymotrypsin, α_{obsd} −0.70°. The aqueous solution was acidified with 0.6 ml. of 1.0 *N* hydrochloric acid, taken to dryness in vacuum and extracted with acetone. The acetone was evaporated and the residue was washed with ether and dried; 0.0624 g. (0.30 mmole), 56% yield, m.p. 193–195°, α_{obsd} −3.10°, $[\alpha]^{22}D$ −175° (0.9% in absolute ethanol), optically active β-acetamido-β-phenylpropionic acid. The infrared spectrum was determined in KBr prism and was identical with that of the synthetic racemic compound.

Anal. Calcd. for $C_{11}H_{13}O_3N$: N, 6.76. Found: N, 6.88.

The original ether extract was washed with water, dried and concentrated in vacuum leaving a residue of the ester (0.813 g., 3.46 mmoles), 80% yield. This was dissolved in chloroform (5 ml.), treated with charcoal, filtered and examined in the polarimeter, α_{obsd} +1.93°, $[\alpha]^{22}D$ +38.5°, calculated for 22% hydrolysis. The infrared spectrum was identical with that of the racemic starting material.

Diethyl α-Acetamidomethylmalonate.—Diethyl ethoxymethylenemalonate (75 g., 0.35 mole, Kay–Fries) was shaken with 180 ml. of concentrated ammonia, an oil separating, which slowly crystallized. The ammonia was decanted and the crystals were washed with ice-water and with hexane; 46 g. (0.25 mole), 70% yield, diethyl aminomethylenemalonate, m.p. 64–65.5° from ether–petroleum ether, reported[15] 67°. Attempted hydrogenation of a portion of this over platinum oxide in acetic anhydride solvent failed. Diethyl aminomethylenemalonate (30 g., 0.161 mole), was boiled under reflux for 6 hr. with 300 ml. of acetic anhydride and concentrated in vacuum. The residue was triturated and washed well with water; **diethyl acetamidomethylenemalonate** (24.6 g., 0.107 mole), 67% yield, m.p. 56° from petroleum ether.

Anal. Calcd. for $C_{10}H_{15}NO_5$: C, 52.40; H, 6.55; N, 6.11. Found: C, 52.5; H, 6.4; N, 6.2.

A portion of this product (6.87 g., 0.030 mole) was hydrogenated in 50 ml. of ethanol over 0.2 g. of platinum oxide,

(13) T. B. Johnson and J. E. Livak, *J. Am. Chem. Soc.*, **58**, 299 (1936).

(14) G. Stefanovic, S. Mihajlovic and M. Stefanovic, *J. Org. Chem.*, **18**, 1467 (1953).

(15) L. Claisen, *Ann.*, **297**, 1 (1897).

833 ml. of hydrogen being absorbed in 100 minutes. The solution was filtered and concentrated leading to diethyl α-acetamidomethylmalonate (6.75 g., 0.028 mole), 93% yield, m.p. 38–38.5° from hexane–ether.

Anal. Calcd. for $C_{10}H_{17}NO_5$: C, 51.94; H, 7.36; N, 6.06. Found: C, 51.94; H, 7.43; N, 6.22.

Dipotassium α-Acetamidomethylmalonate.—A solution of 1.15 g. (0.005 mole) of diethyl α-acetamidomethylmalonate in 2 ml. of ethanol was treated with 22.6 ml. of 0.464 *N* potassium hydroxide (0.0104 mole) in ethanol. A precipitate was collected after 4 hr. and washed with ethanol; 1.20 g. (0.0043 mole), 86% yield, dec. 285°.

Anal. Calcd. for $C_6H_7NO_5K_2 \cdot 2H_2O$: C, 25.08; H, 3.48; N, 4.88. Found: C, 24.38; H, 3.65; N, 5.32.

Ethyl Hydrogen α-Acetamidomethylmalonate.—A solution of 4.62 g. (0.020 mole) of diethyl α-acetamidomethylmalonate in 80 ml. of ethanol was treated gradually with stirring over a period of 3 hr. with 41.0 ml. of 0.464 *N* potassium hydroxide (0.019 mole) in ethanol. The solution was evaporated to dryness at room temperature, and the residue was washed with ether. The residue was dissolved in a little water, 19.0 ml. of 1 *N* hydrochloric acid was added, the solution was concentrated to dryness at 1 mm., and the residue was extracted with acetone. The extract was concentrated to dryness, and the oily residue was treated with hexane, slowly crystallizing, giving the monoester (3.75 g., 0.0185 mole), 92% yield, melting 65–75° dec. This was converted to a derivative:

1-(Ethyl α-acetamidomethylmalonyl)-1,3-bis-(*p*-dimethylaminophenyl)-urea.—Ethyl hydrogen α-acetamidomethylmalonate (1.0 g., 4.9 mmoles) in 10 ml. of acetone was treated with 1.2 g. (4.3 mmoles) of 1,3-bis-(dimethylaminophenyl)-carbodiimide[16] in 20 ml. of acetone for 20 hr. atr oom temperature. The solution was decanted from a precipitate and evaporated to form a solid residue (1.64 g., 3.4 mmoles), 80% yield, m.p. 153–154° dec. from benzene. The infrared spectrum was determined in chloroform: 2.89(w), 3.06(w), 3.35(m), 5.78(s), 5.94(s), 6.17(m), 6.57(s), 6.90(w), 7.38-(m), 7.57(w), 8.13(m), 10.60(m) μ.

Anal. Calcd. for $C_{25}H_{33}N_5O_6$: C, 62.11; H, 6.83; N, 14.49. Found: C, 61.65; H, 6.85; N, 14.00.

Hydrolysis of Diethyl α-Acetamidomethylmalonate by α-Chymotrypsin.—(1) A solution of 0.1159 g. (0.500 mmole) of the ester in 15 ml. of water and 2 ml. of 0.1 *M* Na_2HPO_4 buffer was placed in the *p*H stat at *p*H 8.1 and found not to hydrolyze. Enzyme was added, 0.1036 g., and 0.2 *N* sodium hydroxide was added automatically, 2.17 ml. (0.434 mmole) being consumed in 22 hr., 87% reaction. The polarimeter reading was the same as that of the α-chymotrypsin alone.

(2) A solution of 0.922 g. (4.00 mmoles) of the ester and 0.200 g. of the enzyme in 20 ml. of water and 0.5 ml. of buffer was allowed to react in the *p*H stat, 3.076 ml. of 1.0 *N* sodium hydroxide being consumed in 21 hr., 77% reaction, α_{obsd} −0.88°, possible contribution due to hydrolysis products +0.23°. The solution was taken to dryness in vacuum, and the residue was washed with ether to remove unreacted ester. The residue was dissolved in water, treated with 3.0 ml. of 1.0 *N* hydrochloric acid and taken to dryness again. The residue was extracted with acetone, which was concentrated, leading to a residue which was crystallized from chloroform–hexane; ethyl hydrogen α-acetamidomethylmalonate, 0.617 g. (3.04 mmoles), 99% yield, m.p. 70–80° dec. This was dissolved in chloroform, treated with Norit A, filtered, diluted to 10 ml. and examined in the polarimeter, α_{obsd} +0.03°. The chloroform was evaporated, and the residue was triturated with hexane and dried, 0.587 g. being recovered. A portion of this (0.503 g., 2.48 mmoles) was dissolved in 3 ml. of water, brought to *p*H 7 with 2.27 ml. of 1.0 *N* sodium hydroxide, filtered and examined in the polarimeter, α_{obsd} −0.06°. The solution was reacidified with 2.1 ml. of 1.0 *N* hydrochloric acid, taken to dryness and extracted with acetone leading to recovery of 0.484 g. (2.38 mmoles) of the monoester. This was dissolved in 4 ml. of acetone and treated with 0.586 g. (2.10 mmoles) of 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide in 10 ml. of acetone for 20 hr. The solution was filtered and concentrated, leading to the ureide (0.790 g., 1.64 mmoles), 78% yield, crystallized from benzene, m.p. and mixed m.p. with an authentic sample, 153–154°. A portion, 0.544 g., was dissolved in 5 ml. of chloroform and examined in the polarimeter, α_{obsd} 0.00°. The infrared spectrum was identical with that of the compound obtained in the non-enzymatic hydrolysis.

(3) A solution of 0.795 g. (3.44 mmoles) of the ester IV, 0.104 g. of α-chymotrypsin and 1.5 ml. of buffer in 22 ml. of water was allowed to react at *p*H 7.8, 0.466 ml. of *N* NaOH being consumed in 210 min., 13.5% reaction, 0.821 ml. in 420 min., 23.9% reaction. The solution was examined in the polarimeter, α_{obsd} −0.49 ± 0.02°, α_{obsd} for α-chymotrypsin blank, −0.47 ± 0.02°.

Diethyl α-Benzyl-α-acetamidomalonate.—Sodium (1.2 g., 0.05 mole) was dissolved in 75 ml. of absolute ethanol, and 10.9 g. (0.05 mole) of diethyl acetamidomalonate was added, followed by 6.9 g. (0.055 mole) of benzyl chloride. The mixture was boiled for 4 hr., concentrated in vacuum to about 15 ml. and treated with 100 ml. of 0.5 *M* acetic acid. Crystals were collected, washed with cold water, dried and recrystallized from benzene–ethanol; 6.6 g. (0.021 mole), 43% yield, m.p. 105–105.5°, reported[17] 106°.

A suspension of 0.307 g. (1 mmole) of the ester in a solution of 2.0 ml. of 0.1 *M* Na_2HPO_4 and 0.300 g. of α-chymotrypsin in 20 ml. of water was brought to *p*H 7.8, stirred magnetically and observed in the *p*H stat. There was essentially no consumption of alkali.

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Requirements for Stereospecificity in Hydrolysis by α-Chymotrypsin. IV. The Hydroxyl Substituent. Absolute Configurations[1]

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dl-Ethyl lactate (I), diethyl α-hydroxymalonate (II), *dl*-ethyl β-hydroxybutyrate (III), *dl*-ethyl β-phenyl-β-hydroxypropionate (IV), dimethyl β-hydroxyglutarate (V) and diethyl β-hydroxyglutarate (VI) have been hydrolyzed by α-chymotrypsin. Compounds I, II and III were hydrolyzed with no stereospecificity, compound III hydrolyzing very slowly. Compounds IV, V and VI were hydrolyzed stereospecifically, leading to optically pure (−)-β-phenyl-β-hydroxypropionic acid and the (−)-monoalkyl hydrogen β-hydroxyglutarates, which were characterized as their 1,3-bis-(*p*-dimethylaminophenyl)-ureides. The stereospecific hydrolyses of V and VI and of the previously studied diethyl β-acetamidoglutarate proceed in the L-sense. The effectiveness of alpha and beta hydroxyl and acetamido groups in leading to stereospecific hydrolysis by α-chymotrypsin is compared.

Study of the hydrolysis of α-chymotrypsin of a number of esters containing α- and β-acetamido substituents—diethyl α-acetamidomalonate,[2] diethyl β-acetamidoglutarate,[3] ethyl α-acetamido-

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