those of native phycoerythrins do not react at all. The native proteins mask the pyrrole nitrogens of the phycoerythrobilins, possibly through direct hydrogen bonding. By breaking these bonds, urea quenches the fluorescence of the chromophores and exposes their pyrrole nitrogens to zinc ions.

The spectra of cryptomonad and B-phycoerythrins, which differ greatly in neutral aqueous solution,[5] are similar in acid and 8 *M* urea solutions (λ_{max} at 557 and 540 mμ, respectively). Phycoerythrobilin is the only pigment obtained from these two chromoproteins, but there are probably many phycoerythrobilin units per protein molecule.[6] The single absorption maximum of cryptomonad phycoerythrin indicates that its phycoerythrobilin–protein attachments are of one kind, while the two-peaked spectrum of native B-phycoerythrin indicates that some of its phycoerythrobilins are bound differently from the rest, depending, presumably, on the neighboring protein environment. The 544 mμ peak of native B-phycoerythrin corresponds to that of the urea-denatured chromoprotein and may be attributed to non-hydrogen bonded, non-fluorescent chromophores, the 566 mμ maximum being due to hydrogen bonded, fluorescent chromophores.

At wave lengths longer than 530 mμ, the spectrum of R-phycoerythrin, whether in water, acid or urea solutions, is similar to that of B-phycoerythrin and, therefore, we consider that its 568 and 542 mμ maxima are attributable to hydrogen bonded and non-hydrogen bonded phycoerythrobilins, respectively. The 497 mμ maximum of R-phycoerythrin, unlike those at 542 and 568 mμ, is not shifted on denaturation, but is shifted when zinc acetate is added to the native protein (Fig. 2), and, since it behaves independently of the phycoerythrobilin maxima, it must be attributed to a different pigment. These results also indicate that the pyrrole nitrogens of the 497 mμ chromophore are not masked by hydrogen bonding with the native protein.

Hydrolysis studies indicate that the 497 mμ chromophore is a urobilinoid pigment.[11] Both isomerization and oxidation of phycoerythrobilin yield such pigments, whose zinc complexes correspond in their absorption maxima to the 497 mμ chromophore-zinc-protein maximum at 512 mμ. That there are two independent chromophores on R-phycoerythrin is also indicated by the fluorescence spectra of the zinc complex of the denatured chromoprotein, which displayed a maximum at 520 mμ when excited at $\lambda_{490\ m\mu}$, and at 600 mμ when excited at $\lambda_{540\ m\mu}$.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, BRANDEIS UNIVERSITY, WALTHAM 54, MASSACHUSETTS]

Requirements for Stereospecificity in Hydrolysis by α-Chymotrypsin. Diethyl β-Acetamidoglutarate[1]

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Diethyl β-acetamidoglutarate has been prepared and hydrolyzed by α-chymotrypsin. Lacking the structural features of typical substrates for this enzyme, it is hydrolyzed slowly but asymmetrically, leading to (+)-ethyl hydrogen-β-acetamidoglutarate, $[\alpha]^{25}D$ + 5.9°, which has also been prepared by resolution of the *dl* compound. Experiments with inhibitors indicate that this hydrolysis, and the similar asymmetric hydrolysis of diethyl-α-acetamidomalonate, are caused by the one active site of the enzyme. Association of substrate with enzyme via the characteristic structural features, which when present may lead to high reactivity and chemical specificity, is not required for and may not be responsible for stereospecificity.

Introduction

Consideration of the specificity of enzymatic reactions indicates that it may be desirable to elucidate two separate, but perhaps not entirely independent, sets of factors: first, those elements of structure in the substrate which contribute to high reactivity and are responsible for chemical specificity, and, second, those structural features which, by diastereomeric or conformational interactions with groups on the enzyme, lead to stereospecificity.[2] This may imply that high reactivity and stereospecificity are determined neither at identical sites on the enzyme nor at the same time. However, the stereospecificity of an enzyme frequently is shown by its causing one enantiomorph of a pair to undergo reaction very rapidly and the other very slowly or not at all and is thus intimately related to measurement of chemical reactivity. Thus a distinction may fail to be made between the two sets of factors and they may be thought to be one and the same, the requirements for stereospecificity, in effect, not being subjected to independent investigation.

Attention was especially centered on enzyme stereospecificity some years ago when it was found that symmetric molecules of type Ca,b,d,d could be formed, enzymatically, asymmetrically labelled and could undergo enzymatic reaction asymmetrically at the two sites *d*.[3] The explanation of this in terms of three point contact[4] was plausible,

(1) (a) We are pleased to acknowledge support of this work by the Division of Research Grants, The National Institutes of Health, RG 4584. (b) For a preliminary report, see S. G. Cohen and E. Khedouri, *Nature*, **186**, 75 (1960).

(2) S. G. Cohen and L. H. Klee, THIS JOURNAL, **82**, 6038 (1960).

(3) For references on this subject, see ref. 2.

(4) A. G. Ogston, *Nature*, **162**, 963 (1948).

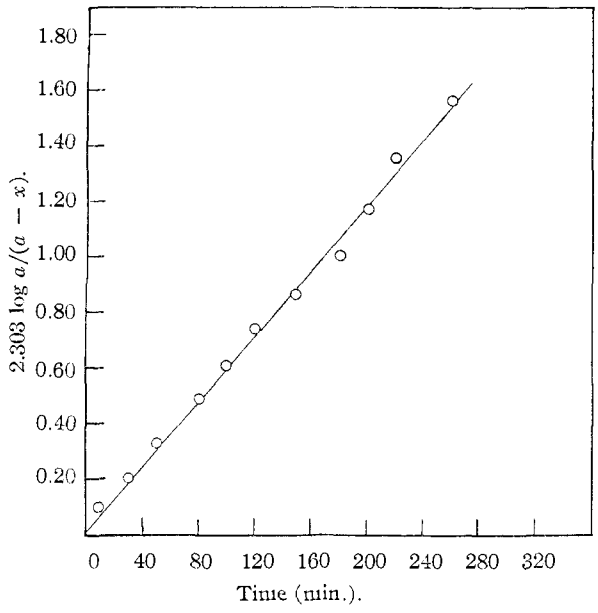

Fig. 1.—Hydrolysis of 0.0434 *M* diethyl β-acetamidoglutarate by α-chymotrypsin (10 mg./ml.), 27°, *p*H 7.8.

particularly in view of the earlier proposal of multisite contact[5] in enzyme reactions, and has been widely accepted both for the situation for which it was proposed and for enzyme stereospecificity in general.[6] Thus, when the structural features for a typical highly reactive substrate for α-chymotrypsin were elucidated—a beta-aryl group, an alpha-acylamido and the ester or other group being hydrolyzed,[7] all combining to provide an L-configuration at the alpha-carbon—it appeared plausible that these were the three groups providing the three point contact and leading to stereospecificity, and that if one or more were missing, reactions would be both slow and non-stereospecific.[8] Testing the possibility that high reactivity and stereospecificity were influenced or determined *separately* in an enzyme catalyzed reaction course and granting that one or more of the same functional groups contributed to both effects, we have studied the action of chymotrypsin on several substrates. We have reported[2] previously on the hydrolysis by α-chymotrypsin of diethyl α-acetamidomalonate, DEAM, a substrate which lacks the β-aryl group which is characteristic of typical substrates for this enzyme. We found that the reactivity was rather low but that the reaction proceeded asymmetrically, leading to optically active ethyl sodium α-acetamidomalonate. The β-aryl group was thus not required for stereospecificity, a result foreshadowed in the observations that Michaelis-constants and inhibitor constants for α-chymotrypsin may be independent of stereochemistry, providing that the β-aryl group is present,[7] and consistent with the fact that the β-aryl group does not lead to stereospecificity in the hydrolysis of ethyl β-phenyl-α-chloropropionate.[9]

(5) M. Bergmann and J. S. Fruton, *Advances in Enzymology*, **1**, 63 (1941).

(6) K. J. Laidler, "The Chemical Kinetics of Enzyme Action," Oxford Press, New York, N. Y., 1958, pp. 172–173.

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

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

(9) J. E. Snoke and H. Neurath, *Arch. Biochem.*, **21**, 351 (1949).

Results

We are now reporting results of the hydrolysis by α-chymotrypsin of diethyl β-acetamidoglutarate, DEAG, which lacks both the structural features characteristic of typical substrates for this enzyme. DEAG was prepared by the following series of reactions: condensation of diethylmalonate with chloroform, leading to glutaconic acid; conversion of this to diethyl glutaconate; addition of ammonia and acetylation to form DEAG. *dl*-Ethyl hydrogen-β-acetamidoglutarate was prepared by hydrolysis of DEAG to β-acetamidoglutaric acid by barium hydroxide, conversion of the diacid to the cyclic anhydride by treatment with acetic anhydride, and conversion of the anhydride, by treatment with ethanol, to the racemic ethyl hydrogen-β-acetamidoglutarate (±)-MEAG, m.p. 77–78°.

The hydrolysis of DEAG by α-chymotrypsin was followed at 27°, *p*H 7.8, by use of a *p*H meter, standard alkali being added manually to maintain *p*H. The substrate was stable at this *p*H in the absence of enzyme, and the enzymatic reaction was quite slow, equal weights of enzyme and substrate being used to provide a convenient rate. The concentration of enzyme used was 10 mg./ml. The rate of hydrolysis was reproducible and the reaction showed kinetics first order in the ester with a half life of about 116 minutes under these conditions, k_1 = 0.0057 min.$^{-1}$ (Fig. 1). Systematic variations in substrate concentration were not made in this work, and we have no evidence as to whether the Michaelis–Menten equation is applicable, the primary purpose being to ascertain whether the reaction was stereospecific. Consumption of alkali slowed down after 80–85% hydrolysis of one ester group; the reaction then was interrupted and the solution was worked up for the product. The solution was acidified, taken to dryness in vacuum and extracted with acetone, leading in 79% yield to optically active ethyl hydrogen-acetamidoglutarate, (+)-MEAG, m.p. 92–93°, $[\alpha]^{25}D$ + 5.9°. Alternatively, the same product was isolated after dialysis of the reaction solution. Infrared spectra of the active and *dl* compounds were identical. (+)-MEAG was obtained as the only product of the reaction, and its melting point and specific rotation were not changed by further crystallization, indicating that the hydrolysis was completely stereospecific. Further confirmation of this was obtained by resolution of the synthetic (±) MEAG, a procedure we were unable to apply in the hydrolysis of diethyl α-acetamidomalonate,[2] which led to the easily racemized malonate half-ester. Treatment of (±)-MEAG with cinchonidine led to a diastereomeric salt, which, after crystallization to constant melting point and rotation, was decomposed to form (+)-MEAG, m.p. 92–93°, $[\alpha]^{25}D$ 5.8°, identical with that from the enzyme catalyzed hydrolysis. Further treatment of residues with strychnine led to a diastereomeric salt, which, after repeated crystallization, was decomposed to form (−)-MEAG, m.p. 93°, $[\alpha]^{25}D$ −5.9°.

Since a very high concentration of α-chymotrypsin was used in these experiments to furnish a convenient rate, some experiments were carried out with inhibitors which are normally effective in diminish-

ing the rate or stopping the hydrolysis of typical substrates to assure that the same active site is utilized in hydrolysis of the substrates which lacked the structural features characteristic for this enzyme. The experiments were not such as to allow a kinetic analysis, but the following results were observed. In parallel experiments in the presence and absence of hydrocinnamic acid, this inhibitor reduced the rate of hydrolysis of diethyl β-acetamidoglutarate to about one-third and reduced the initial rate of hydrolysis of diethyl α-acetamidomalonate to about 50–60% of that observed in its absence. In each case the weight of hydrocinnamic acid was three times that of the enzyme; the concentrations of substrate and inhibitor (I) were (a) DEAG, 0.0178 *M*, (I) 0.088 *M*; (b) DEAM, 0.054 *M*, (I) 0.030 *M*. In addition striking inhibition was shown when the enzyme was pretreated with 1.5 molar equivalents of diisopropylphosphofluoridate (DFP). DEAG showed some hydrolysis initially, which then stopped, apparently as the stirring completed phosphorylation and the poisoning of the enzyme. Hydrolysis of DEAM was also markedly inhibited by DFP, occurring at about 5% of the uninhibited rate, when corrected for the non-enzymatic hydrolysis, which was about half that of the inhibited rate.

Conclusions

Typical substrates for α-chymotrypsin have as characteristic structural features beta-aryl and alpha-acylamido groups. Such structures lead to high reactivity, to chemical specificity, when the stereochemistry is correct. Diethyl β-acetamidoglutarate, lacking the characteristic structural features of a typical substrate for α-chymotrypsin but possessing acetamido and carbethoxymethylene substituents at the beta position, is hydrolyzed slowly but apparently completely asymmetrically by this enzyme. Inhibitor studies indicate that the hydrolysis depends upon the single active site of the enzyme. The beta-aryl group, when present, is involved in formation of the initial complex[2] and thus may contribute to high chemical reactivity, but this interaction neither depends on correct stereochemistry[7] nor does it lead alone to stereospecificity.[9] DEAM and DEAG may not form such a complex effectively, if at all,[2] and are attacked slowly by the enzyme. Their stereospecific hydrolyses may result from diastereomeric interactions of the groups about the developing center of asymmetry in the substrate with an asymmetric grouping at the active site of the enzyme. It may be that the acylamido group—alpha in DEAM, beta in DEAG—is particularly important in these interactions, but the other groups, both the hydrolyzing function and the second ester groups, may also be involved in these interactions, affecting the energy of the transition states and the stereospecificity. Study of models shows that as the functional group of an asymmetric reagent attacks one group d of Ca,b,d,d, other groups of the reagent interact differently with the groups a and b at the developing center of asymmetry C, depending upon which of the groups d is attacked. Similar conformational interactions may also occur, subsequent to the initial complexing, with the typical substrates, which do form enzyme-substrate complexes readily. In these cases, it seems likely that three point contact, involving in a gross way the three characteristic structural features of typical substrates for chymotrypsin, is not the determinant of stereospecificity; nor is such contact involved in the stereospecific reactions of the molecules of type Ca,b,d,d which we have studied—DEAM and DEAG. The importance of the acylamido group and the second ester group for stereospecific hydrolysis of these substrates, the significance of the spatial relations of such groups to the hydrolyzing function and to the center, or developing center of asymmetry, and the absolute configuration of the stereospecificity are now under investigation.

Experimental[10]

Diethyl β-Acetamidoglutarate, DEAG.—Diethyl glutaconate was prepared by the procedure described for the dimethyl ester.[11] Diethyl malonate, 128 g. (0.80 mole), was condensed with 65 g. (0.50 mole) of chloroform in 700 ml. of ethanol in the presence of 136 g. (2.0 mole) of sodium ethoxide, leading to the disodio derivative of diethyl α,α′-dicarboethoxy-β-chloro-glutarate, and thence on treatment with 5% hydrochloric acid to the free acid. This was boiled for 3 hr. with 800 ml. of 3:1 6 *N* hydrochloric acid:ethanol and concentrated in vacuum, leading to glutaconic acid, m.p. 136–138°, reported[11] 138°. The acid was boiled for 24 hr. in excess absolute ethanol and 2 ml. of sulfuric acid, concentrated, neutralized with sodium bicarbonate, extracted with ether, dried and distilled, leading to diethyl glutaconate, b.p. 120–125° (20–25 mm.), reported[12] 119–120° (15 mm.), 50 g. (0.27 mole), over-all yield 68%. Diethyl β-aminoglutarate was prepared by treatment of diethyl glutaconate, 50 g. (0.27 mole) with 200 ml. of absolute ethanol saturated with dry ammonia; the temperature was kept at 50–55° and ammonia gas was passed through for 36 hr. The solution was concentrated in vacuum, the residue was extracted with ether and the extract was concentrated leading to diethyl β-aminoglutarate, 36 g. (0.17 mole), 65% yield. The c ude β-amino ester, 36 g. (0.17 mole), was heated for 1 hr. on the water-bath with 70 ml. of acetic anhydride and 36 g. of fused sodium acetate, concentrated in vacuum and extracted with ether. The extract was concentrated and distilled in vacuum leading to diethyl β-acetamidoglutarate, 40 g. (0.16 mole), 93% yield, b.p. 198–203° (25 mm.).

Anal. Calcd for $C_{11}H_{19}NO_5$: C, 53.88; H, 7.81; N, 5.71. Found: C, 54.33; H, 7.84; N, 5.50. Infrared absorption peaks were observed at 2.95 μ(w), 3.38(w), 5.78(s), 5.97(m), 6.65(m), 7.25(m), 7.70(m), 9.80(w).

β-Acetamidoglutaric Acid.—Diethyl β-acetamidoglutarate, 5.0 g. (0.020 mole), was boiled for 3 hr. in a solution of 7.5 g. (0.024 mole) of $Ba(OH)_2 \cdot 8H_2O$ in 50 ml. of water. Barium was precipitated by addition of 2.34 g. of sulfuric acid, the mixture was filtered, the filtrate was taken to dryness in vacuum, the residue was extracted repeatedly with absolute ethanol, the extracts were concentrated leading to β-acetamidoglutaric acid, m.p. 185–186° (dec.), from ethanol, 3.65 g. (0.019 mole), 95% yield. *Anal.* Calcd. for $C_7H_{11}NO_5$: N, 7.41. Found: N, 7.38; sol. water; sl. sol. ethanol; insol. chloroform, ether, acetone. In the residue, not dissolved in the ethanol extraction, was apparently found β-aminoglutaric acid, decomposing 265–270°, identified as the hydrochloride, m.p. 290–305°, reported[12] 290–295°.

β-Acetamidoglutaric Anhydride.—β-Acetamidoglutaric acid, 2.8 g. (0.015 mole), was heated at 100° for 0.5 hr. in 28 ml. of acetic anhydride, the solution was concentrated in vacuum and the residue was treated with Norite in acetone and crystallized from acetone–ether, 2.5 g., 94% yield, m.p. 140–141°.

(10) Infrared spectra were obtained in chloroform on a Perkin-Elmer model 21 spectrophotometer. Melting points are uncorrected. Elementary analyses are by Dr. S. M. Nagy, Massachusetts Institute of Technology.

(11) E. P. Kohler and G. H. Reid, THIS JOURNAL, **47**, 2807 (1925).

(12) H. Feuer and W. A. Swartz, *ibid.*, **77**, 5427 (1955).

Anal. Calcd. for $C_7H_9NO_4$: C, 49.11; H, 5.30; N, 8.18. Found: C, 49.05; H, 5.26; N, 8.16.

***dl*-Ethyl Hydrogen-β-acetamidoglutarate.**—β-Acetamidoglutaric anhydride, 1.5 g. (0.0088 mole) was boiled for 6 hr. in 20 ml. of ethanol, the solution was concentrated in vacuum and the residue was crystallized from acetone–ether, m.p. 77–78°, 1.05 g. (0.0049 mole), 56% yield.

Anal. Calcd. for $C_9H_{15}NO_5$: C, 49.73; H, 6.96; N, 6.45. Found: C, 49.71; H, 6.92; N, 6.42. Ins. ether; sol. chloroform, acetone, alcohol.

The infrared absorption spectrum was similar to that of the diester, showing absorption bands at 2.95 μ (w), 3.29 (w), 3.45 (w), 5.80 (s), 6.00 (s), 6.65 (m), 7.10 (m), 7.30 (m), 7.70 (w), 8.55 (m), 9.00 (m), 9.75 (m).

***dl*-Methyl hydrogen-β-acetamidoglutarate** was prepared from the anhydride and methanol in 72% yield as described above, m.p. 131–132°, from acetone.

Anal. Calcd. for $C_8H_{13}NO_5$: C, 47.23; H, 6.45; N, 6.89. Found: C, 47.45; H, 6.44; N, 7.01.

Hydrolysis of DEAG by α-Chymotrypsin. (+)-Ethyl Hydrogen-β-acetamidoglutarate. K5.—A solution of 0.533 g. (2.17 mmole) of DEAG, 1.5 ml. of *N* Na_2HPO_4 and 0.9 ml. of *N* NaH_2PO_4, 0.530 g. of α-chymotrypsin and 50 ml. of water was brought to *p*H 7.8 with 2.21 ml. of 0.428 *N* sodium hydroxide. The hydrolysis was followed with a *p*H meter at 27° under nitrogen, measured volumes of the standard alkali being added to maintain *p*H, 1.80 meq., 83% of one equivalent being required in 5 hr. The solution was brought to *p*H 2 with dilute hydrochloric acid and taken to dryness in vacuum; the residue was extracted 10 times with acetone, the acetone was evaporated, leaving 0.325 g., 69%, of crude product. This was taken up in chloroform, treated with charcoal, concentrated and treated with ether, leading to a solid which was crystallized from acetone–ether, 0.267 g. (1.23 mmole), 57% yield (68% corrected for 83% reaction), of (+)-monoethyl β-acetamidoglutarate, m.p. 92–93°, $\alpha_{obsd.}$ + 0.33°, 2.8% in acetone, $[\alpha]^{25}D$ + 5.9°.

Anal. Found: C, 49.80; H, 6.92; N, 6.51.

The melting range of a mixture with the *dl* compound (of m.p. 77–78°) was 75–91°. In another similar run (K7), with less manipulation, the yield was 66% (79% corrected), m.p. 92–93°, $\alpha_{obsd.}$ + 0.64°, 5.5% in acetone, $[\alpha]^{25}D$ + 5.9°. In another run the hydrolysate was dialyzed, and the product was isolated from the dialysate, $[\alpha]^{25}D$ + 5.9°.

(−)-Cinchonidine (+)-Ethyl Hydrogen-β-acetamidoglutarate.—Equimolar quantities of the active half acid (from chymotrypsin hydrolysis) and the alkaloid, 7.5 and 10.2 mg., respectively, were dissolved in 0.5 ml. of hot ethyl acetate and the solution was cooled. The first crystals and the residue obtained by evaporation of the mother liquor were identical, m.p. 124°, $\alpha_{obsd.}$ −0.62°, 0.375% in chloroform, $[\alpha]^{26}D$ −83°.

Resolution of *dl*-Ethyl-hydrogen-β-acetamidoglutarate.—Equimolar quantities of the *dl*-half acid and cinchonidine, 5.75 g. and 7.82 g., respectively, were dissolved in 110 ml. of hot ethyl acetate, and the solution was refrigerated overnight, leading to crystals of the partially resolved salt of the (+) enantiomorph. The crystals were recrystallized four times from ethyl acetate leading to the cinchonidine salt of constant m.p. and rotation, 1.42 g., 21% yield, m.p. and mixed m.p. with the sample above, 124°, $[\alpha]^{21}D$ − 83°, 0.375% in chloroform.

Anal. Calcd. for $C_{28}H_{37}N_3O_5$: C, 65.73; H, 7.29. Found: C, 65.3; H, 7.0. A portion of the salt, 0.320 g., was dissolved in 20 ml. of dilute hydrochloric acid saturated with sodium sulfate and extracted with ethyl acetate leading to (+)-ethyl hydrogen-β-acetamidoglutarate, 0.112 g., m.p. and mixed m.p. with a sample from the α-chymotrypsin hydrolysis, 92–93°, $\alpha_{obsd.}$ + 0.29°, 2.5% in acetone, $[\alpha]^{25}D$ + 5.8.

The original filtrate was taken down to dryness, leaving a solid residue, 8.5 g., melting 110–130°. This was decomposed as described above and the recovered half ester was crystallized from acetone–ether leading to partially resolved (−)-ethyl hydrogen-β-acetamidoglutarate, 2.65 g., melting 78–94°, $[\alpha]^{25}D$ −1.8°. An equimolar mixture of this ester and strychnine, 2.60 g. and 4.00 g., respectively, was crystallized five times from chloroform–ether to constant rotation, $[\alpha]^{25}D$ −51.8°, 0.375%, chloroform, 0.90 g., 13% yield, melting unsharply at 105°. This salt was decomposed as described above, leading to (−)-ethyl hydrogen-β-acetamidoglutarate, 0.275 g., m.p. 93°, mixed m.p. with the (+) enantiomorph, 77–78°, $\alpha_{obsd.}$ −0.295°, 2.5% in acetone, $[\alpha]^{25}D$ −5.9°.

Anal. Found: C, 49.3; H, 6.8; N, 6.4.

Inhibition by Hydrocinnamic Acid.—The relative proportions of enzyme and inhibitor were those reported in a study of the hydrolysis of ethyl lactate.[8]

1. **Diethyl β-Acetamidoglutarate.**—A solution of 0.109 g. (0.445 mmole) of the ester, 0.110 g. of α-chymotrypsin, 0.330 g. (2.20 mmole) of hydrocinnamic acid in 25 ml. of water containing 1 ml. of 0.1 *N* Na_2HPO_4 was followed in a *p*H stat with magnetic stirring, *p*H 7.9, 32°. The extents of hydrolysis observed were: 1 hr., 13.1%; 2 hr., 18.6%; 3 hr., 23.5%; 4 hr., 26.8%; 5 hr., 30.4%. A solution without inhibitor, containing 0.105 g. of the ester and 0.105 g. of the enzyme in 25 ml. of water containing 1 ml. of 0.1 *N* Na_2HPO_4, was observed similarly with these results: 0.5 hr., 24.8%; 1 hr. 34.2%; 4 hr. 68%.

2. **Diethyl α-Acetamidomalonate.**—A solution of 0.217 g. (1.00 mmole) of this ester, 0.0274 g. of α-chymotrypsin, 0.0822 g. (0.547 mmole) of hydrocinnamic acid, 1 ml. of 0.1 *N* buffer in a total volume of 18.2 ml. was followed in the *p*H stat, *p*H 7.8, 32°. The extents of hydrolysis observed were: 10 minutes, 26.1%; 15 minutes, 41.5%; 20 minutes, 50%; 30 minutes, 66%; 60 minutes, 89%. A solution without inhibitor, containing 0.217 g. of this ester and 0.0274 g. of α-chymotrypsin and buffer in a total volume of 18 ml. showed the following extents of hydrolysis: 4 minutes, 18.6%; 10 minutes, 44.4%; 15 minutes, 60.5%; 20 minutes, 72%; 30 minutes, 86%; 40 minutes, 92%.

Inhibition by Diisopropyl Phosphofluoridate (DFP).—Experiments were carried out in 0.2 *M* phosphate buffer with the relative proportions of enzyme and DFP used in the literature.[13] DFP was provided by Dr. Helen Vanvunakis as a 0.1 *M* solution in 2-propanol and was diluted to 0.001 *M* in the buffer.

1. **Diethyl β-Acetamidoglutarate.**—A solution of α-chymotrypsin in the buffer was treated with DFP, stored for 20 minutes and then used: the ester, 0.105 g., was added to 22 ml. of buffer solution containing 0.105 g. of α-chymotrypsin and 0.00614 mmole of DFP, and the hydrolysis was followed in a *p*H stat at 31.5°, initially at *p*H 7.5, and after 0.5 hr., at *p*H 7.8. After 1 hr., 8.6% hydrolysis was observed, after 2 hr., 9.3%, 4 hr., 10.2%.

2. **Diethyl α-Acetamidomalonate.**—A solution of α-chymotrypsin in the buffer was treated with the DFP, stored at 4° for 6 hr., *p*H 7.3 and then stirred for 20 minutes. The ester, 0.217 g., was added to this solution of 0.0274 g. of enzyme and 0.00160 mmole of DFP in 18 ml. of the buffer, and the hydrolysis was observed in the *p*H stat, *p*H 7.8, 32° as follows: 30 minutes, 15%; 55 minutes, 30%; 80 minutes, 43%. A blank run was carried out at *p*H 7.8 in the 0.2 *M* phosphate buffer, in the absence of enzyme and poison with these results: 30 minutes, 7%; 55 minutes, 14%; 80 minutes, 22%. The blank in the presence of enzyme is described in the preceding set of experiments.

(13) A. K. Balls and E. F. Jansen, *Advances in Enzymology*, **13**, 321 (1952).