alanyl-L-cysteinylglycine ethyl ester[22,31] (XII) and the cleavage of oxytocin. The dehydroalanine peptides resulting from the elimination reaction were not isolated.

Cleavage of XII.—A solution of 24.3 mg. of XII (50 μmoles) in 10 ml. of 60% ethanol was treated with 37.2 mg. (200 μmoles) of FDNB. The reaction was carried out at pH 6 and was followed with a pH-Stat, 0.492 ml. of 0.1 *N* sodium hydroxide being consumed. This is equivalent to a 98% yield. The excess of FDNB was allowed to react with 250 μmoles of thioglycolic acid at pH 8. Ethyl acetate (50 ml.) was then added and the mixture was washed with 0.1 *N* sodium bicarbonate to remove the S-dinitrophenylated thioglycolic acid. The ethyl acetate layer was dried over Na_2SO_4 and was concentrated to 25 ml.

The elimination reaction was carried out on a 4-ml. aliquot (8 μmoles). The sample was dried *in vacuo* at 40° and was treated with 1 ml. of 0.1 *N* sodium methoxide. After 3 min. the extent of elimination was 99% as determined spectrophotometrically. Aliquots of 0.25 ml. (2 μmoles) were taken for cleavage.

(a) **Hydrolytic Cleavage.**—The pH of the sample was adjusted to 7 with dilute hydrochloric acid and the mixture was lyophilized; 1 ml. of 0.01 *N* hydrochloric acid was added, and the tube containing the reaction mixture was closed and heated to 100° for 1 hr. The reaction mixture was lyophilized, and then 1 ml. of 0.1 *N* sodium hydroxide and 0.2 ml. of 30% hydrogen peroxide were added and the reaction mixture was incubated at 37° for 30 min. The reaction mixture was then neutralized with acetic acid and excess of hydrogen peroxide was destroyed by incubation with catalase (0.03 ml. of 5% solution). Aliquots were taken for high voltage electrophoresis. Only one amino acid glycine was detected and the yield was 0.94 μmole (47%).

(b) **Oxidative Cleavage with Bromine.**—The pH of the 0.25-ml. sample was adjusted to 5 with dilute acetic acid and 0.2 ml. of 0.02 *N* aqueous bromine (2 μmoles) was added. After 5-min. incubation at room temperature the pH was adjusted to 7 with dilute sodium hydroxide, and 0.2 ml. of 30% hydrogen peroxide in 0.5 ml. of 0.2 *N* sodium hydroxide was added. The reaction mixture was treated as described above, the yield of the liberated glycine being 0.9 μmole (45%).

(c) **Oxidative Cleavage with Performic Acid.**—The neutralized sample was lyophilized, and the residue was dissolved at 0° in 0.3 ml. of performic acid and kept at this temperature for 2 hr. The performic acid was removed by lyophilization and the residue was dissolved in 1 ml. of 0.1 *N* sodium hydroxide. Hydrogen peroxide 30% (0.2 ml.) was added. The reaction mixture was treated as described above; the yield of glycine detected after electrophoresis was 1.26 μmoles (63%).

Cleavage of Oxytocin.—A 1.8-mg. sample of lyophilized oxytocin (obtained from 90 ampoules of Syntocinon, Sandoz Ltd., Basel) was dissolved in 5 ml. of water. Redistilled thioglycolic acid (2.76 mg., 0.03 mmole) was added and the pH was adjusted to 8.6 with 5% trimethylamine. The container was flushed with nitrogen and the solution was allowed to stand overnight at room temperature. Dinitrophenylation was carried out at room temperature by adding 37.2 mg. of fluorodinitrobenzene (0.2 mmole), dissolved in 0.5 ml. of ethanol, and the pH was maintained at 8.6 for 1 hr. The excess of fluorodinitrobenzene was treated with an excess of thioglycolic acid (46 mg., 0.5 mmole) at the same pH. The reaction mixture was transferred to a separatory funnel and was extracted twice with 20-ml. portions of ethyl acetate. The dinitrophenylated reduced oxytocin which was suspended in the organic layer was washed with 1 *N* potassium bicarbonate in order to remove the excess thioglycolic acid and S-DNP-thioglycolic acid. The ethyl acetate solution was evaporated *in vacuo* at 40°. The yellow residue, found to be insoluble in the usual organic solvents, was suspended in 0.25 ml. of ethanol, and the elimination reaction was carried out by heating to 80° for 1 min., after adding 2 ml. of 0.1 *N* sodium hydroxide. A small amount of unreacted starting material was recovered by centrifugation and again treated as above. The amount of dinitrothiophenolate ion produced was estimated by measuring the difference spectrum at 408 mμ of an aliquot of nonoxidized solution against an equal aliquot which had been oxidized completely with 0.05 ml. of 0.1 *N* iodine. A total amount of 2.75 μmoles of dinitrothiophenolate ion was found corresponding to 1.37 μmoles of oxytocin. The pH of the combined reaction mixture was adjusted to 6.1 with dilute acetic acid and the solution was lyophilized.

The cleavage of the modified oxytocin was carried out by dissolving the lyophilized residue in 1 ml. of performic acid at 0°. A 0.1-ml. aliquot was removed and used as a control sample. The reaction mixture was incubated for 2 hr. at 0° and then the performic acid was removed by lyophilization. The oxidized product was dissolved in dilute alkali, and the reaction mixture was treated with 0.2 ml. of 30% hydrogen peroxide. Sodium hydroxide was then added to a final concentration of 0.1 *N*, and the solution was incubated for 30 min. at 37°. The reaction mixture was then neutralized with acetic acid. The excess of hydrogen peroxide was destroyed by incubation with catalase (0.03 ml. of a 5% solution). The final volume was 5.5 ml.

The amount of N-terminal proline released was determined on a 1-ml. aliquot using proline iminopeptidase as described by Sarid, *et al.*[32] By this method, 0.186 μmole (75%) of free proline was found. Under the same conditions, no free proline could be found in a control sample (which had undergone the same treatment).

Another 1-ml. aliquot was dinitrophenylated according to the method of Levy.[33] The dinitrophenylated product was subjected to acid hydrolysis in a sealed tube with constant boiling hydrochloric acid at 105° for 16 hr. An aliquot of the hydrolysate was developed by ascending chromatography in the "toluene" solvent in one dimension and by descending chromatography with 1.5 *M* phosphate buffer in the second dimension. Only two spots corresponding to di-DNP-tyrosine and 2,4-dinitroaniline could be detected on the chromatogram. The di-DNP-tyrosine spot was eluted from the chromatogram and estimated spectrophotometrically at 360 mμ; 0.098 μmole (40%) was found.

Acknowledgments.—Many thanks are due to Professor E. Katchalski and Professor A. Berger for helpful discussions and to Mrs. H. Later for competent technical assistance.

(31) Dr. I. Photaki, University of Athens, kindly supplied us with a sample of this compound.

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[CONTRIBUTION OF THE DEPARTMENT OF CHEMISTRY, BRANDEIS UNIVERSITY, WALTHAM 54, MASS.]

Kinetics of Hydrolysis of Diethyl Glutarate and β-Substituted Diethyl Glutarates by α-Chymotrypsin[1]

BY SAUL G. COHEN AND JOHN CROSSLEY

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The kinetics of hydrolysis by α-chymotrypsin of four diethyl glutarates, $X-CH(CH_2CO_2Et)_2$, have been studied. The parameters at pH 7.8, 25.0°, 0.1 *M* NaCl, are: X = HO, K_m = 0.097 mole/l., k_3 = 0.032 sec.$^{-1}$; X = CH_3CONH, K_m = 0.065, k_3 = 0.033; X = CH_3COO, K_m = 0.037, k_3 = 0.20; X = H, K_m = 0.016, k_3 = 0.035. The slow stereospecific hydrolyses, X = HO, CH_3CONH, and the more rapid nonstereospecific hydrolyses, X = CH_3COO, H, have been interpreted in terms of associations of: (1) the β-acetamido and β-hydroxyl groups at the α-acylamido enzyme site; (2) the carbethoxyl groups at the β-aryl and hydrolytic sites; (3) the acetoxyl acyl group at the β-aryl site; and (4) hydrogen at a restricted volume site.

Introduction

The symmetric compound diethyl β-hydroxyglutarate, $HO-CH(CH_2CO_2C_2H_5)_2$ (I), was hydrolyzed by α-chymotrypsin,[2] slowly but with high stereospecificity in the L-sense, leading in high yield to the optically active half ester, (−)-HO-CH(CH$_2$COOH)(CH$_2$CO$_2$C$_2$H$_5$). The related asymmetric compound ethyl *dl*-β-hydroxy-

(1) Requirements for stereospecificity in hydrolysis by α-chymotrypsin VI. For paper V see ref. 6. We are pleased to acknowledge generous support of this work by the Division of Research Grants, the National Institutes of Health, RG 4584.

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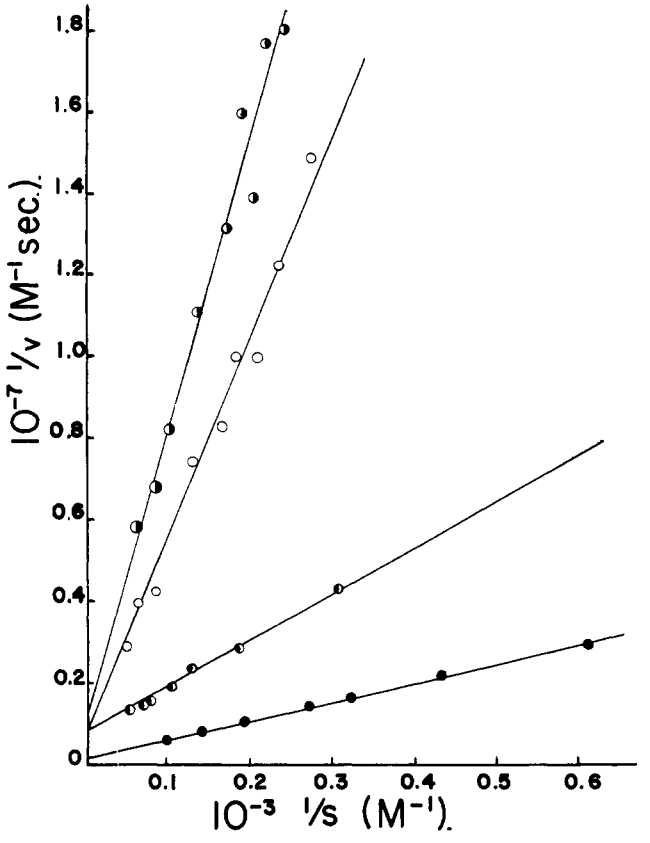

Fig. 1.—Hydrolysis of diethyl glutarates by α-chymotrypsin (1.0 mg./ml., 0.1 *M* NaCl, pH 7.8, 25.0°): —◑—◑—◑—, diethyl β-hydroxyglutarate; —○—○—○—, diethyl β-acetamidoglutarate; —◐—◐—◐—, diethyl glutarate; —●—●—●—, diethyl β-acetoxyglutarate.

butyrate, HO–CH(CH_3)$CH_2CO_2C_2H_5$, was hydrolyzed more slowly than the glutarate and without stereospecificity.[2] The symmetric diethyl β-acetamidoglutarate, CH_3CONH–$CH(CH_2CO_2C_2H_5)_2$ (II), was also hydrolyzed by α-chymotrypsin,[3] slowly and stereospecifically, apparently in the L-sense,[2] leading in high yield to (+)-CH_3CONH–CH$\langle^{CH_2COOH}_{CH_2CO_2C_2H_5}$, while the related asymmetric compound ethyl *dl*-β-acetamidobutyrate, CH_3CONH–$CH(CH_3)CH_2CO_2C_2H_5$, was not hydrolyzed.[4] These symmetric compounds appear to have a greater tendency to react stereospecifically rather than requiring a special mechanism. The second carbethoxy group interacts or associates with the enzyme in a manner both to increase the rates of hydrolysis and to favor stereospecificity. In this it appears to function like the β-aryl group of "natural" substrates of this enzyme and it may associate at the β-aryl site. This indicated that diethyl N-acetylaspartate might behave analogously to ethyl N-acetyl-β-phenylalaninate; the L-enantiomorph of the aspartate then proved to be an excellent substrate[5] for α-chymotrypsin.

Diethyl β-acetoxyglutarate, CH_3CO_2–$CH(CH_2CO_2C_2H_5)_2$, III, analogous to I and II but lacking the active proton on the β-substituent, was hydrolyzed by α-chymotrypsin with *no* stereospecificity[6] leading in high yield to the inactive half ester

(±)-CH_3CO_2–CH$\langle^{CH_2CO_2H}_{CH_2CO_2C_2H_5}$

Furthermore, this hydrolysis appeared to be considerably more rapid[7] than the stereospecific hydrolyses of I and II. The unsubstituted diethyl glutarate IV was also found to be hydrolyzed by α-chymotrypsin,[7] necessarily without reference to stereospecificity, but more rapidly than I and II. In this structurally related set of glutarate esters, stereospecificity, which may be generally thought of as a rate-enhancing property, was associated with decreased reactivity. It seemed of interest to examine in more detail these slow stereospecific and more rapid non-stereospecific hydrolyses.

Results and Discussion

The hydrolysis by α-chymotrypsin of the β-substituted diethyl glutarates I, II, and III, isolation of the half esters, and characterization of these as the ureides derived from treatment with 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide have been described previously.[2,6] Diethyl glutarate itself has now been hydrolyzed by α-chymotrypsin, the reaction stopping after hydrolysis of one ester group; the half ester so produced was isolated and characterized as the substituted ureide which was identical with an authentic sample.

The rates of hydrolysis of the four diethyl glutarates (0.006 m./l.) by α-chymotrypsin (1 mg./ml.) were compared briefly in 0.1 *M* NaCl at pH 7.8. The hydrolyses showed apparent first-order kinetics to the extent that they were followed. The order of reactivity was found to be: β-CH_3COO– > β-H > β-CH_3CONH– > β-HO–, and the relative rates were approximately 10:4.5:1.7:1. The results are summarized in Table I.

TABLE I

HYDROLYSIS OF DIETHYL GLUTARATES, X–$CH(CH_2CO_2C_2H_5)_2$, 0.006 *M*, BY α-CHYMOTRYPSIN, 1 MG./ML., 0.1 *M* NaCl, pH 7.8, 25.0°

Time, min.	Reaction, %			
	X = HO–	CH_3CONH–	CH_3COO–	H
30	2.1	3.8	23.4	10.0
60	4.5	7.5	41.8	19.2
90	6.9	11.0	..	26.8
120	9.4	15.5	..	33.3
150	11.9	21.6	..	..
210	..	28.7	..	..
280	..	36.3	..	..

We have reported[3] previously that the hydrolysis of diethyl β-acetamidoglutarate by α-chymotrypsin was retarded by hydrocinnamic acid and strongly inhibited by diisopropyl phosphofluoridate, indicating that this hydrolysis was probably being effected by the usual active site. The specific inhibitor diphenylcarbamyl chloride[8] has now been found to inhibit essentially completely hydrolysis by α-chymotrypsin of the four diethyl glutarates I, II, III, and IV, indicating that the active site which leads to hydrolysis of the natural substrates is probably also involved in the hydrolysis of the glutarates.

The kinetics of hydrolysis of the four diethyl glutarates were studied at pH 7.8, 0.1 *M* NaCl, catalyzed by 1.0 mg./ml. of α-chymotrypsin. The procedure is described in the Experimental section. Pseudo-zero-order kinetics were observed for the initial rates over a range of concentrations of each substrate and these are listed in Table II. Linear double reciprocal plots[9] ($1/V$ *vs.* $1/(S)$) were obtained and are drawn in Fig.

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TABLE II

RATES OF HYDROLYSIS OF DIETHYL GLUTARATES, X-CH-($CH_2CO_2C_2H_5$)$_2$, BY α-CHYMOTRYPSIN (1.0 MG./ML.), 0.1 *M* NaCl, pH 7.8, 25.0°

X	$S \times 10^3$, mole/l.	$V \times 10^7$, mole/l./sec.	X	$S \times 10^3$, mole/l.	$V \times 10^7$, mole/l./sec.
HO	4.25	0.55	CH_3COO	1.63	3.38
	4.63	.56		2.30	4.59
	4.95	.72		3.07	6.30
	5.45	.63		3.64	7.05
	6.01	.76		5.08	9.53
	7.55	.90		7.08	12.40
	10.01	1.22		9.67	18.75
	11.80	1.48	H	3.19	2.31
	16.27	1.74		5.28	3.48
CH_3CONH	3.74	0.67		7.64	4.16
	4.21	.81		9.57	5.12
	4.68	.98		12.35	6.11
	5.42	.95		14.27	6.50
	6.16	1.19		17.48	7.19
	7.52	1.34			
	9.79	1.47			
	12.10	2.33			
	16.25	2.53			
	20.55	3.30			

1. Least-squares analyses of these data led to values of the kinetic parameters K_m and k_3 for each substrate, and these results are given in Table III.

TABLE III

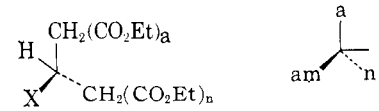
KINETIC CONSTANTS FOR HYDROLYSES OF DIETHYL GLUTARATES, X-CH($CH_2CO_2C_2H_5$)$_2$, BY α-CHYMOTRYPSIN, 0.1 *M* NaCl, pH 7.8, 25.0°

X	K_m, mole/l.	k_3, sec.$^{-1}$
HO–	0.097[a]	0.032[a]
CH_3CONH–	.065	.033
CH_3COO–	.037	.20
H–	.016	.035

[a] Errors in K_m and k_3 are estimated as ±10% of the tabulated values.

The β-hydroxy- and β-acetamidoglutarates I and II, which had been hydrolyzed stereospecifically, had low rates which were reflected in less favorable K_m and k_3. The β-acetoxyglutarate III, which had been hydrolyzed without stereospecificity, had the highest rate, reflecting a more favorable K_m and a far more favorable k_3. Diethyl glutarate itself, hydrolyzing more rapidly than I and II but less rapidly than III, had its high reactivity due solely to its most favorable K_m, all β-substituents leading to less favorable K_m. As an approximation K_m may be taken in part as an index of the effectiveness of binding of the substrate to the enzyme, k_3 as an index of the rate of hydrolytic attack of the enzyme on the bound substrate.

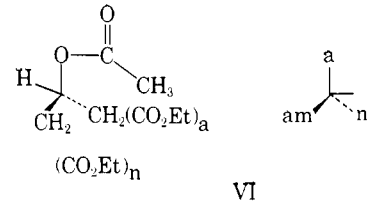
Association of the substrates with this enzyme may be considered in terms of the groups, tetrahedrally oriented about the developing center of asymmetry at the β-carbon atom, interacting with complementarily oriented sites on the enzyme.[10-12] The latter may be designated as a, the β-aryl site; am, the acylamido site; and n, the nucleophilic or hydrolytic site, the character of the sites referring to the substituents on the natural substrates for this enzyme. The fourth site, not designated, is one of restricted volume which accommodates the fourth substituent, the hydrogen atom,[6,12] in all substrates. An association of the diethyl glutarates with α-chymotrypsin may be indicated schematically in V.



V

Comparison of the glutarates and butyrates[2-4] and consideration of the rapid hydrolysis of diethyl N-acetylaspartate[5] indicate that the second carbethoxyl group, $(CO_2Et)_a$, associates at the β-aryl site, a, much like the β-phenyl in β-phenylalanine, but not as firmly. This association makes it possible for the substituent X to associate effectively at the α-acylamido site, am. When this substituent is acetamido or hydroxyl, interaction, presumably by hydrogen-bonding, takes place at this site, and, the hydrogen taking its normal position, stereospecific hydrolyses occurs in the L-sense. However, these associations leave the hydrolyzing carbethoxyl, $(CO_2Et)_n$, one methylene group further removed from the nucleophilic site than it is in other substrates, *i.e.*, ethyl N-acetylphenylalaninate and diethyl N-acetylaspartate, in which the acylamido group is α to the hydrolyzing carbethoxyl group. This may have two effects: (1) it decreases effective association with the hydrolytic site of the enzyme through this carbethoxyl group, which is important,[13] and this increases K_m; (2) it requires a distortion of the enzyme for the hydrolytic site to act on the ester group, leading to low k_3. In this way stereospecific but slow hydrolyses of substrates I and II result.

However the acetoxyl substituent, lacking a polar hydrogen, does not seem to associate effectively with the acylamido site and apparently prefers to associate at the β-aryl site, a. Evidence for this is seen in the hydrolysis of ethyl α-acetoxypropionate by α-chymotrypsin, in which the D-enantiomorph hydrolyzes more rapidly than the L,[6,12] although the nitrogen analog, ethyl N-acetylalaninate, shows normal L-stereospecificity. This inversion of normal stereospecificity was ascribed to association of the acetoxyl–acyl group at the β-aryl site.[6,12] Thus in the glutarate, when X is acetoxyl, both the carbethoxyl and the acetoxyl groups may, separately, associate at the β-aryl site, a. When the carbethoxyl does, the interaction indicated in V occurs and leads to hydrolysis of $-(CO_2Et)_n$; when the acetoxyl–acyl group associates at a, the interaction indicated in VI occurs and leads to hydrolysis of the other carbethoxyl, $-(CO_2Et)_a$, by the hydrolytic site, n. Diethyl β-acetoxyglutarate may associate thus



VI

in two nearly equivalent ways with the enzyme, leading to hydrolysis of one or the other carbethoxyl groups; diminished stereospecificity would be expected and none is found. However, this complete absence of stereospecificity might not be expected. Since the acetoxyl and carbethoxyl groups do not associate well at the acylamido site am in interactions V and VI, respectively, the substrate is not as constrained as in the associations of the hydroxy- and acetamidoglutarates with the enzyme, and the hydrolyzing group

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may more readily associate at the hydrolytic site, n. Effective associations of the two carbethoxyls at a and n in V and the acetoxyl at a and carbethoxyl at n in VI lead to more favorable K_m and k_3. In these associations the groups $-C(=O)CH_3$ and $-C(=O)O-R$ seem to resemble the phenyl group and associate at the β-aryl site, but the distance between these groups and the hydrolyzing ester group is not optimum for this enzyme.

In diethyl glutarate itself, IV, the substituent X is H and the two carbethoxyl groups now become sterically indistinguishable; in V, either may be associated with a while the other is at n, the identity of the "associating" hydrogen changing. However, in this case it is not clear whether the methylene α or β to the hydrolyzing ester group will fulfill this function. In any event the two carbethoxyl groups associate effectively at a and n leading to the most favorable K_m. However, k_3 is lower and the over-all reactivity of this substrate is less than that of the acetoxy, but greater than those of the hydroxy- and acetamidoglutarates. Natural substrates for α-chymotrypsin do have an additional substituent—the acylamido group—and the acetoxyl group in III may assist the enzyme in adopting a more reactive conformation.

Experimental

Melting points are uncorrected. Elementary analyses are by D. C. Daessle, Montreal, Canada.

Diethyl β-hydroxyglutarate was obtained from Chemical Procurement Laboratories and distilled before use; b.p. 100–101° (0.35 mm.), $n^{26}D$ 1.4358; reported[14] b.p. 105–107° (2 mm.), $n^{20}D$ 1.4381. **Diethyl β-acetamidoglutarate** had been prepared previously[3]; a sample was redistilled; b.p. 132–135° (0.1 mm.), $n^{23}D$ 1.4541. Diethyl β-hydroxyglutarate (10 g., Chemicals Procurement Laboratories) was heated under reflux for 1.5 hr. with 20 g. of acetic anhydride, concentrated, and distilled under vacuum, leading to **diethyl β-acetoxyglutarate**, b.p. 108° (0.1 mm.), $n^{26}D$ 1.4295, reported[15] b.p. 138° (4 mm.). The infrared spectrum indicated the absence of olefin. **Diethyl glutarate** was obtained from Eastman Kodak Co. and distilled before use; b.p. 123° (11 mm.), $n^{25}D$ 1.4238.

α-Chymotrypsin was obtained from Worthington Biochemical Corp., 3× recrystallized, salt free. The molecular weight was assumed to be 25,000.

1-(4-Carbethoxybutanoyl)-1,3-bis-(dimethylaminophenyl)-urea.—Glutaric acid (5.0 g. 0.038 mole) was boiled under reflux in 40 ml. of acetic anhydride for 1 hr., concentrated, and cooled, leading to glutaric anhydride (3.0 g., 0.026 mole), 67% yield, m.p. 55° from benzene–ethanol, reported[16] m.p. 55°. The anhydride (2.8 g., 0.024 mole) was suspended in 30 ml. of absolute ethanol and heated on the steam bath for 4 hr. The solution was concentrated and the residue was distilled, leading to ethyl hydrogen glutarate (2.0 g., 0.013 mole), 51% yield, b.p. 139–140° (3 mm.), $n^{24}D$ 1.4329, reported[17] b.p. 143–145° (7 mm.). The half ester (0.2 g., 1.25 mmoles) was dissolved in 20 ml. of acetone and treated with 0.4 g. (1.4 mmoles) of 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide[18] in 20 ml. of ether under reflux for 3 hr., cooled, and filtered, leading to the ureide (0.54 g., 1.22 mmoles), 98% yield, m.p. 157–158° from acetone.

Anal. Calcd. for $C_{24}H_{32}N_4O_4$: C, 65.50; H, 7.25. Found: C, 65.43; H, 7.20.

Hydrolysis of Diethyl Glutarate by α-Chymotrypsin.—Diethyl glutarate (0.97 g., 5.2 mmoles) was added to 20 ml. of 0.1 *M* sodium chloride containing 0.075 g. of α-chymotrypsin, and consumption of 1 *N* alkali was followed in a pH Stat, pH 7.2, 25°, under nitrogen, with magnetic stirring. Readings of time in hours and ml. of 1 *N* alkali are: 7.0, 1.23; 23, 3.57; 30, 4.26; 48, 5.08. The reaction essentially stopped at 98% hydrolysis of one ester group. The solution was brought to pH 3 with 5 *N* HCl and evaporated to dryness at room temperature. The residue was extracted with acetone, the extract was filtered and concentrated, and the oil residue was treated with chloroform and concentrated, leading to ethyl hydrogen glutarate; 0.85 g. (5.2 mmoles). The infrared spectrum, taken as a liquid film was identical with that of a synthetic sample. The half ester (0.43 g., 2.7 mmoles) was dissolved in 20 ml. of acetone and treated with 0.80 g. (2.8 mmoles) of 1,3-bis-(*p*-dimethylaminophenyl)-carbodiimide[18] in 20 ml. of ether under reflux for 3 hr., leading to the ureide (0.84 g., 1.9 mmoles), 70% yield, m.p. 157.5–158.5°, from acetone, mixture m.p. with a synthesized sample, unchanged. The infrared spectra of the two samples, taken in Nujol, were identical.

Hydrolyses of Compounds I, II, III, and IV (Table I).—In each case sufficient substrate was added to 20 ml. of 0.1 *M* NaCl in the reaction vessel to provide approximately 0.006 m./l. of substrate. The weights of substrate and their molarity were I, 0.0242 g., 5.93×10^{-3} mole/l.; II, 0.0292, 5.97×10^{-3}; III, 0.0295, 5.97×10^{-3}; IV, 0.0224, 5.94×10^{-3}. The solutions were brought to pH 7.8, and the pH was maintained by a Radiometer Titrator Model TTT1b which delivered 0.1 *N* NaOH from an Aminco automatic buret. The solutions were kept under nitrogen at 25.0 ± 0.1° and stirred magnetically. Hydrolysis of each substrate by water at this pH was followed and these correction factors were determined and applied to the subsequent rates determined in the presence of enzyme. α-Chymotrypsin (0.020 g., dried weight) was added and readings of alkali uptake *vs.* time were taken, corrected, and plotted in the first-order way. The data at uniform time intervals, Table I, were taken from these graphs.

Inhibition by Diphenylcarbamyl Chloride.—In each blank experiment a solution of 20 mg. of α-chymotrypsin (0.80×10^{-6} mole) in 20 ml. of 0.1 *M* NaCl was prepared, 0.1 ml. of methanol was added, a weighed quantity of substrate was added, and the rate of consumption of alkali was followed for 15–20 min. at pH 7.8 as described above, a zero-order rate being observed. In each inhibited run, a solution of 20 mg. of α-chymotrypsin in 20 ml. of 0.1 *N* NaCl was prepared, a solution of 0.1 ml. of methanol containing 1.85×10^{-4} g. (0.80×10^{-6} mole) of diphenylcarbanyl chloride was added, and the whole was stirred for 20 min. The substrate (at the same concentration as before) was then added; in all cases no enzyme-catalyzed hydrolysis occurred.

Kinetic Procedure.—Reactions were carried out in the pH-Stat described above, pH 7.8, 25.0 ± 0.1°, in a 30-ml. beaker containing 20 ml. of 0.1 *M* NaCl and equipped with a circulating water jacket, Teflon-coated magnet stirrer, and a Lucite cover through which passed the electrodes, the buret tip, a nitrogen inlet tube, and sample holders. Nitrogen was passed through distilled water and over the solution, stirring was begun, the ester was dropped in, and the rate of nonenzymatic hydrolysis was followed for 25 min. The α-chymotrypsin, 0.020 g., was added, the solution was brought back to pH 7.8 and the uptake of 0.1 *N* sodium hydroxide was followed for 20–30 min. After correction for nonenzymatic hydrolysis and for consumption of alkali by the enzyme, plots of added alkali *vs.* time were linear. The results are summarized in Table II.

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