$[\alpha]^{26}D$ −54.7° (*c* 0.17, 10% acetic acid); single Pauly-, Sakaguchi-, and Ehrlich-positive, ninhydrin-negative spot on paper chromatography, R_f^1 0.61, and on paper electrophoresis in pyridinium acetate pH 3.5; amino acid ratios in acid hydrolysate, $ser_{2.17}tyr_{0.96}but_{1.13}glu_{1.00}his_{1.04}phe_{1.09}arg_{3.01}gly_{1.91}lys_{2.80}pro_{2.00}val_{1.91}$, MSH activity *in vitro* 2.2 × 10^6 MSH units/g.

Seryltyrosylseryl-α-amino-*n*-butyrylglutaminylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginylprolylvaline Amide Acetate Hydrate (XXXI).—The protected eicosapeptide amide XXX (45 mg.) was dissolved in anhydrous trifluoroacetic acid (0.6 ml.) and the solution was kept at room temperature for 20 min. Water (4 ml.) was added and the solution was immediately frozen and lyophilized. The residue was dissolved in water (15 ml.). Amberlite IRA-400 (acetate cycle) (approximately 3 ml. settled in water) was added, and the suspension was stirred for 50 min. at room temperature. The mixture was filtered, the resin was washed with several portions of water, and the filtrate and washings were concentrated to a small volume *in vacuo* and then lyophilized, giving a colorless fluffy powder; yield 41.8 mg. This material was dissolved in water (30 ml.) and the solution was applied to a CMC column (1.5 × 20 cm.) which was eluted successively with the following ammonium acetate buffers: pH 6.8, 0.075 *M* (30 ml.); pH 6.8, 0.25 *M* (40 ml.); pH 8.5, 0.25 *M* (40 ml.); pH 9.0, 0.25 *M* (100 ml.); pH 9.5, 0.025 *M* (100 ml.); and pH 10.0, 0.25 *M* (100 ml.). Individual fractions, 5 ml. each, were collected at a flow rate of approximately 2 ml. per min. Absorbancy at 280 mμ located the desired material in the pH 9.5, 0.25 *M* eluates (tubes 71–109) which were pooled, the bulk of the solvent was removed *in vacuo*, and the concentrate was lyophilized to constant weight, giving a colorless fluffy powder; yield 25.6 mg., $[\alpha]^{28}D$ −61.7° (*c* 0.09, 10% acetic acid); single ninhydrin-, Pauly-, Sakaguchi-, and Ehrlich-positive spot with R_f^3 0.7 × His; single band on disk electrophoresis on polyacrylamide gel[25] at pH 4.6; amino acid ratios in acid hydrolysate, $ser_{1.97}tyr_{0.91}but_{0.94}glu_{1.02}his_{1.02}phe_{0.98}arg_{2.91}gly_{1.89}lys_{3.31}pro_{2.16}val_{1.89}$; acid ratios in LAP digest, $ser_{2.1}tyr_{1.00}but_{0.0} > glu_{0.74}his_{0.57}phe_{0.65}arg_{0.78}try_{0.48}gly_{0.52}lys+orn_{1.04}pro_{0.70}val_{0.57}$; 0.26 μmole of peptide liberated 0.16 μmole of glutamine on digestion with Pronase, glutamic acid was not detectable in the digest on paper chromatograms or on the amino acid analyzer; the peptide did not liberate amino acids on treatment with carboxypeptidase A; MSH activity[34] 1.6 × 10^7 MSH units/g.; adrenal ascorbic acid depleting activity i.v. 31.2 ± 4.2 to 48.4 ± 10.4 IU/mg.

Pronase Experiments.—Pronase (Calbiochem. Lot. No. 502117, 45000 P.U.K./g.) (0.1 mg.) in 0.025 *M* ammonium acetate buffer, pH 7.4 (0.1 ml.), was incubated for 24 hr. at 40° with a solution of peptide XXXI (1.5–2.5 mg.) in 0.025 *M* ammonium acetate buffer, pH 7.4 (0.1 ml.). Digestion was stopped by evaporating the solution to dryness *in vacuo* over P_2O_5 and KOH. The residue was dissolved in 0.2 *N* sodium citrate buffer, pH 2.2, for amino acid analysis.

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

Kinetics of Hydrolysis of Dicarboxylic Esters and Their α-Acetamido Derivatives by α-Chymotrypsin[1]

BY SAUL G. COHEN AND JOHN CROSSLEY

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The diethyl esters of malonic, succinic, glutaric, α-acetamidomalonic, N-acetylaspartic, and N-acetylglutamic acids are hydrolyzed to the monoesters by α-chymotrypsin, the latter three stereospecifically to the α- acids. The kinetic parameters K_m and k_3 have been determined for each ester. Values of k_3/K_m for the six compounds are, respectively, 1, 7.5, 1.7, 23, 950, and 64, the succinate hydrolyzing most rapidly of the unsubstituted compounds, the aspartate most rapidly of the α-acetamido compounds. The second carbethoxyl group associates at the β-aryl site of the enzyme, most effectively, leading to lowest K_m, when β relative to the hydrolyzing group. Its effectiveness is compared with other β-substituents. The α-acetamido group leads to increased reactivity by increasing k_3.

Introduction

Esters of glutaric acid and of β-substituted glutaric acids,[2] $RO_2CCH_2CHXCH_2CO_2R$, X = H, HO, CH_3CONH, and CH_3CO_2, are hydrolyzed by α-chymotrypsin,[2] the β-hydroxy[3] and the β-acetamido[4] compounds stereospecifically, and the β-acetoxy compound more rapidly but nonstereospecifically.[2,5] The unsubstituted glutarate itself was hydrolyzed more readily than the β-hydroxy and the β-acetamido compounds. The corresponding derivatives of butyric acid are inert or may be hydrolyzed exceedingly slowly and apparently without stereospecificity.[3,6] The second carbethoxyl group in the glutarates, like the β-aryl group in "natural" substrates of this enzyme, contributes to reactivity and stereospecificity in these reactions. Dicarboxylic esters may provide a group of relatively water-soluble substrates in which a significant structure–reactivity relation may be studied in an enzymatic reaction, and it seemed of interest to examine a series of unsubstituted and α-acetamido-substituted diesters. Diethyl N-acetylaspartate, the analog in this set of ethyl N-acetyl-β-phenylalaninate, was examined first and found to be hydrolyzed by α-chymotrypsin with high effectiveness and stereospecificity.[7] We are now reporting on the kinetics of the enzymatic hydrolysis of the diethyl esters of malonic, succinic, glutaric, adipic, α-acetamidomalonic, and N-acetylglutamic acids. Diethyl α-acetamidomalonate had been reported previously to be hydrolyzed stereospecifically by α-chymotrypsin.[8]

Results

Preparative experiments were carried out first to establish whether all the compounds would, in fact, be hydrolyzed by α-chymotrypsin and to allow characterization of the products. Diethyl malonate, 0.6 g., was hydrolyzed at pH 7.2 by 0.1 g. of the enzyme, 73%

(1) We are pleased to acknowledge generous support of this work by the Division of Research Grants, National Institutes of Health, Grant No. GM 04584.

(2) S. G. Cohen and J. Crossley, *J. Am. Chem. Soc.*, **86**, 1217 (1964).

(3) S. G. Cohen and E. Khedouri, *ibid.*, **83**, 4228 (1961).

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

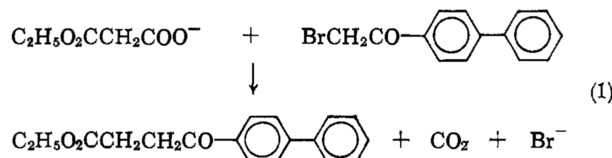
(5) S. G. Cohen, J. Crossley, E. Khedouri, R. Zand, and L. H. Klee, *ibid.*, **85**, 1685 (1963).

(6) S. G. Cohen, Y. Sprinzak, and E. Khedouri, *ibid.*, **83**, 4225 (1961).

(7) S. G. Cohen, J. Crossley, and E. Khedouri, *Biochemistry*, **2**, 820 (1963).

(8) (a) S. G. Cohen and L. Altschul, *Nature*, **183**, 1678 (1959); (b) S. G. Cohen and L. H. Klee, *J. Am. Chem. Soc.*, **82**, 6038 (1960).

of one ester group hydrolyzing in 20 hr., leading to ethyl hydrogen malonate in 87% yield, identical with a synthesized sample. Attempts to prepare a ureide by treatment with 1,3-bis(*p*-dimethylaminophenyl)carbodiimide[3,9] failed. Previous attempts to prepare similar derivatives from ethyl hydrogen α-hydroxymalonate[3] and ethyl hydrogen α-acetoxymalonate[5] had also failed. Treatment with α-bromo-*p*-phenylacetophenone led to a satisfactory derivative, identical with that prepared from a synthesized sample of ethyl hydrogen malonate. This, however, was not the expected phenylphenacyl ester of ethyl malonic acid but, apparently, ethyl 3-*p*-phenylbenzoylpropionate, decarboxylation having occurred at some stage of the preparation of the derivative.



(1)

Diethyl succinate (0.3 g.) was hydrolyzed at pH 7.2 by 0.05 g. of α-chymotrypsin, one ester group hydrolyzing essentially quantitatively in 4 hr., the reaction then stopping. Ethyl hydrogen succinate was isolated quantitatively and characterized as the substituted ureide by treatment with 1,3-bis(*p*-dimethylaminophenyl)carbodiimide, which was identical with that prepared from a synthesized sample of the half-ester. Diethyl glutarate (0.7 g.) was hydrolyzed at pH 7.2 by 0.5 g. of α-chymotrypsin, 77% of one ester group hydrolyzing in 24 hr., leading to ethyl hydrogen glutarate. The product and its substituted ureide were identical with the previously prepared compounds.[2] Diethyl adipate was not hydrolyzed by α-chymotrypsin.

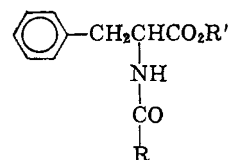
L-(−)-Diethyl N-acetylglutamate was prepared by conversion of L-glutamic acid to L-(+)-diethyl glutamate hydrochloride, followed by acetylation with acetic anhydride, $[\alpha]^{21}D$ −20.3°. The DL- and D-(+)-compounds were prepared similarly. A sample of L-(+)-γ-ethyl-α-hydrogen N-acetylglutamate, $[\alpha]^{21}D$ +4.67°, was prepared by acetylation of commercially available L-γ-ethyl-α-hydrogen glutamate. L-(−)-Diethyl N-acetylglutamate (0.66 g.) was hydrolyzed at pH 7.2 by 0.020 g. of α-chymotrypsin, 70% of one ester group hydrolyzing in 26 min., leading to L-(+)-γ-ethyl-α-hydrogen N-acetylglutamate in 95% yield, $[\alpha]^{21}D$ +4.90°. DL-Diethyl N-acetylglutamate (1.7 g.) was hydrolyzed stereospecifically by 0.035 g. of α-chymotrypsin, one ester group of one enantiomorph hydrolyzing quantitatively in 2 hr., leading to high yields of recovered unhydrolyzed D-(+)-diethyl N-acetylglutamate, $[\alpha]^{21}D$ +19.5°, and of the hydrolysis product L-(+)-γ-ethyl-α-hydrogen N-acetylglutamate, $[\alpha]^{21}D$ +4.80°. When D-(+)-diethyl N-acetylglutamate was treated with α-chymotrypsin 3% hydrolysis of one ester group appeared to take place in 25 min., less than 4% in 80 min., the reaction essentially stopping. This substrate may have contained a few per cent of the L-enantiomorph, leading to this slight extent of reaction.

The preparative hydrolysis of diethyl α-acetamidomalonate by α-chymotrypsin and isolation of optically active ethyl sodium α-acetamidomalonate[8a] and the preparative hydrolysis of L-(−)-diethyl N-acetylaspartate and isolation and characterization of L-(+)-β-ethyl-α-hydrogen N-acetylaspartate[6] have been described previously.

The kinetics of hydrolysis of the substrates by 0.5 mg./ml. of α-chymotrypsin were studied at pH 7.2, 0.1 *M* NaCl, as described in the Experimental section. The low pH, 7.2, was chosen to minimize the corrections for nonenzymatic hydrolysis. These were 8 and 4% for the substrates which were least rapidly attacked by the enzyme, the malonate and glutarate, respectively, and less for the others. The diethyl esters of malonic, succinic, glutaric, α-acetamidomalonic, L-N-acetylglutamic, and DL-N-acetylglutamic acids were examined. Pseudo-zero-order initial rates of hydrolysis of varying initial concentrations of these esters are given in Table I. Linear double reciprocal plots[10] were obtained. Least-squares analyzis of these data led to values of the kinetic parameters K_m and k_3 for each substrate, and these values are given in Table II.

Discussion

These relative reactivities may be considered from the points of view of the several structural features, keeping in mind generally that derivatives of β-phenylalanine[11] may be considered as models of natural substrates for α-chymotrypsin, and that the enzyme may have tetrahedrally oriented sites so spaced as to accommodate effectively the β-aryl group, the α-acylamido group, the hydrolyzing group, and the α-hydrogen of the substrate.[5,12]



1. Effect of Length of Carbon Chain between the Two Ester Groups.—The increased reactivity and stereospecificity of derivatives of dibasic acids as compared with those of related simple monobasic acids arises from the capacity of the second carbethoxyl group to fit into that enzyme site with which the β-aryl group normally associates. The second carbethoxyl group, −COOR, appears most effective when it may associate precisely where the β-phenyl group, C_6H_5, does, and leave the other three groups well located at their normal sites, *i.e.*, when it is attached to the carbon atom β to the hydrolyzing group. Of the unsubstituted esters, the succinate is most reactive as indicated by the data in Table I and the k_3/K_m ratios in Table II. The α- and β-carbethoxyl groups correspond in relative position to the α-carbethoxyl and β-aryl groups of the natural substrates,[7] leading to most favorable K_m. The glutarate requires an accommodation or distortion of substrate and enzyme to fit the two ester groups to the two sites because of the additional intervening methylene group, and less favorable K_m and k_3 are observed. Association of the second carbethoxyl group may be less firm and offer somewhat more leeway than does association of an aryl group, and the adjustment is made without excessive cost. However, an additional methylene

(9) F. Zetzche and W. Neiger, *Ber.*, **73B**, 467 (1940).

(10) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

(11) H. Neurath and G. W. Schwert, *Chem. Rev.*, **46**, 69 (1950).

(12) (a) G. E. Hein and C. Niemann, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1341 (1961); (b) G. E. Hein and C. Niemann, *J. Am. Chem. Soc.*, **84**, 4495 (1962).

TABLE I

RATES OF HYDROLYSIS OF DICARBOXYLIC ESTERS BY α-CHYMOTRYPSIN (0.50 MG./ML.), 0.1 M NaCl, pH 7.2, 25.0°

Diethyl ester	[S] × 10^3, M	V × 10^7 M. sec.$^{-1}$
Malonate	3.31	0.63
	3.62	0.68
	4.52	0.93
	5.68	0.98
	6.20	1.19
	6.40	1.39
	9.05	1.85
	11.78	2.24
	15.32	2.39
	17.75	2.63
Succinate	2.32	2.73
	3.18	3.48
	3.98	3.96
	4.56	4.55
	5.22	4.76
	7.64	6.20
	10.79	7.46
Glutarate	4.65	1.26
	4.67	1.31
	5.55	1.34
	5.76	1.46
	6.70	1.53
	7.16	1.62
	8.20	1.83
	10.85	2.34
	13.56	2.73
	19.25	2.96
Adipate	5	0
α-Acetamidomalonate	1.38	5.81
	1.61	6.71
	1.83	7.70
	2.07	8.85
	2.36	10.2
	2.88	11.4
	3.84	15.3
	4.88	19.7
	7.33	28.2
	9.48	36.2
L-N-Acetylglutamate	1.09	13.2
	1.34	16.1
	1.44	19.6
	1.66	22.2
	2.14	24.9
	2.48	33.1
	2.56	37.0
	2.99	34.2
	3.35	39.0
	4.26	52.5
	7.80	100.0
DL-N-Acetylglutamate	1.98	11.4
	2.12	11.7
	2.42	13.1
	2.64	14.2
	2.82	16.6
	3.25	17.9
	3.82	22.4
	4.25	24.8
	5.05	27.9
	5.86	32.1
	7.86	38.7

group cannot be accommodated and the adipate is inert. In the malonate, one methylene group must span the distance normally spanned by two, leading probably to distortion of the enzyme, to a considerably less favorable K_m and somewhat lower over-all activity. However, k_3 is most favorable for the malonate, an effect possibly due to the distortion, but more likely related to the high reactivity of malonate esters toward basic hydrolysis due to the electronegative α-carbethoxyl substituent.[13]

TABLE II

KINETIC CONSTANTS FOR HYDROLYSIS OF DICARBOXYLIC ESTERS BY α-CHYMOTRYPSIN (0.50 MG./ML.), 0.1 M NaCl, pH 7.2, 25.0°

Diethyl ester	K_m, M	k_3, sec.$^{-1}$	k_3/K_m
Malonate	0.11	0.11	1.0
Succinate	0.0087	0.065	7.5
Glutarate	0.016	0.027	1.7
α-Acetamidomalonate	0.031	0.71	23
L-N-Acetylaspartate[7]	0.023	22	950
DL-N-Acetylaspartate[7]	0.044	21	
L-N-Acetylglutamate	0.078	5.0	64
DL-N-Acetylglutamate	0.16	4.5	

Similarly, of the acetamido diesters, the L-N-acetylaspartate is by far the most reactive, with favorable K_m and k_3. The L-N-acetylglutamate, like the glutarate, is next in reactivity with somewhat less favorable K_m and k_3. The α-acetamidomalonate, like the malonate, is least reactive, with somewhat anomalous kinetic parameters, k_3 being low but K_m intermediate between the values for the aspartate and glutamate.

2. Effect of the α-Acetamido Group.—In each case k_3/K_m values indicate that the α-acetamido compound was considerably more reactive than its unsubstituted analog with the greatest increase, 126 times, in the most reactive pair, succinate–aspartate, the next greatest, 38 times, in the glutarate–glutamate pair, and the least, 23 times, in the pair of lowest reactivity, the malonates. The increase in reactivity due to the α-acetamido group in the succinate–aspartate and glutarate–glutamate pairs arises entirely from large increases in k_3 (340-fold and 190-fold, respectively), the K_m values in the α-acetamido compounds actually being somewhat less favorable than the corresponding unsubstituted diesters. These increases in K_m may result from distortion, or from a kinetic contribution of k_3 to the values of K_m. The malonate pair again is not consistent with the others and shows increase in rate due to contributions from both K_m and k_3. Apart from this, these results indicate that the α-acetamido group, which may enter into a hydrogen-bonding interaction with the enzyme[14] and which is very effective in leading to stereospecificity[6] and to high reactivity,[12] has these effects with but little influence upon the values for K_m, and in some way activates the system (perhaps by distorting the enzyme) after the forces leading to formation of the complex have had their effect.[12,14]

3. Effect of the Second Ester Group.—The succinate and N-acetylaspartate esters may be compared with other substrates for which kinetic data are available. Diethyl succinate appears to be hydrolyzed somewhat more readily by α-chymotrypsin than is methyl β-phenylpropionate,[15] K_m = 0.0039 M, k_3 = 0.013 sec.$^{-1}$. In this pair the β-carbethoxyl compares favorably with the β-phenyl group, leading to somewhat

(13) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., New York, N. Y., 1940, p. 212; A. Skrabal and E. Singer, *Monatsh.*, **41** 339 (1920).

(14) M. L. Bender and B. W. Turnquest, *J. Am. Chem. Soc.*, **77**, 427 (1955).

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

less favorable K_m, more favorable k_3; the hydrocinnamate was studied, however, under different, and perhaps less favorable, conditions in 20% methanol.

L-Diethyl N-acetylaspartate is about one-fiftieth as active as L-methyl N-acetyl-β-phenylalaninate,[16] K_m = 0.0013 M, k_3 = 53 sec.$^{-1}$, having considerably less favorable K_m and somewhat less favorable k_3. Both compounds were studied in water, but at pH 7.2 and 7.8, respectively. L-Methyl N-acetyl-β-cyclohexylalaninate[16] was also more reactive toward α-chymotrypsin than L-diethyl N-acetylaspartate, the β-cyclohexyl group leading to a most favorable K_m, 0.00019 M, and slightly less favorable k_3, 15 sec.$^{-1}$, than the β-carbethoxyl group. L-Methyl N-acetylleucinate[17] showed over-all reactivity similar to that of L-diethyl N-acetylaspartate, K_m = 0.0029 M, k_3 = 4.6 sec.$^{-1}$, the β-isopropyl group leading to more favorable K_m, less favorable k_3, than the β-carbethoxyl group.

Finally, as had been observed for L- and DL-diethyl N-acetylasparate, the value of k_3 for DL-diethyl N-acetylglutamate is very similar to that of the L-enantiomorph, and the value of K_m for the DL-material is twice that for the L-enantiomorph. The second carbethoxyl leads to favorable K_m and over-all reactivity in these stereospecific hydrolyses, but the D-enantiomorph does not hydrolyze and does not appear to inhibit hydrolysis of the L-. If presence of the D-enantiomorph is neglected, the L-enantiomorph shows quite similar K_m and k_3 when present in DL-material and when present as L-. The second carbethoxyl group does not appear to lead to the very firm association characteristic of aryl groups which allows D-enantiomorphs and simple aromatic compounds to be effective inhibitors.

Experimental

Diethyl malonate (Eastman Kodak Co., 16 g., 0.10 mole, b.p. 79° (6 mm.), n^{27}D 1.4102) was stirred for 0.5 hr. at room temperature with 4 g. (0.10 mole) of sodium hydroxide in 40 ml. of ethanol, neutralized with ethanolic hydrogen chloride, filtered, and concentrated leading to **ethyl hydrogen malonate**, 3.2 g. (0.024 mole), 24% yield, n^{25}D 1.4180, lit.[18] n^{20}D 1.4283. The crude material, 0.50 g. (0.0038 mole), was dissolved in 2.5 ml. of water, neutralized to phenolphthalein with 1 N NaOH, and treated with 0.5 g. (0.0018 mole) of α-bromo-p-phenylacetophenone on the steam bath. Ethanol was added to bring the reagent into solution, and boiling was continued for 2 hr. The solution was cooled, leading to the product, m.p. 80–81° from ethanol, 0.50 g. (0.0018 mole), quantitative yield based on the quantity of p-phenylphenacyl bromide used, and on the assumption that the product was **ethyl 3-p-phenylbenzoylpropionate** (see below). The infrared spectrum in Nujol showed bands (μ): 3.4 (s), 5.7 (m), 5.9 (m), 6.2 (w), 6.8 (m), 7.0 (w), 7.25 (m), 7.65 (w), 7.75 (w), 8.05 (m), 8.35 (m), 8.45 (m), 9.05 (w), 9.7 (w), 9.9 (w), and 10.2 (w).

Diethyl malonate, 0.59 g. (3.7 mmoles), was hydrolyzed at 25°, under nitrogen, by 0.100 g. of α-chymotrypsin in 20 ml. of 0.1 N sodium chloride at pH 7.2 in a pH stat, an automatic buret delivering 1 N sodium hydroxide. Hydrolysis essentially stopped after 20 hr., 2.71 ml. of alkali having been consumed, 73% hydrolysis of one ester group. The solution was extracted with two 25-ml. portions of ether to remove unhydrolyzed ester, brought to pH 3 with 1 N hydrochloric acid, and concentrated. The residue was extracted with three 50-ml. portions of acetone, which were concentrated, leading to **ethyl hydrogen malonate**, 0.31 g. (2.4 mmoles), 87% yield. The infrared spectrum (liquid film) was identical with that of the material prepared by hydrolysis with sodium hydroxide, above. The half-ester was treated with α-bromo-p-phenylacetophenone as described above, leading to the same product, m.p. 80–81°, alone and when mixed with the previous product, and with identical infrared spectrum.

Anal. Calcd. for $C_{18}H_{18}O_3$ (ethyl 3-p-phenylbenzoylpropionate): C, 76.50; H, 6.39. Found: C, 76.36; H, 6.17.

Ethyl Hydrogen Succinate.—Succinic anhydride (Eastman Kodak Co., 10 g. (0.10 mole)) was boiled in 50 ml. of ethanol for 3 hr. and concentrated, and the residue was distilled, leading to the half-ester, 6.8 g. (0.046 mole), 46% yield, b.p. 128–129° (3 mm.), n^{25}D 1.4298, lit.[19] n^{21}D 1.4323. A solution of 0.20 g. (1.4 mmoles) of the half-ester in 20 ml. of dry acetone was treated with 0.40 g. (1.3 mmoles) of 1,3-bis(p-dimethylaminophenyl)carbodiimide[3,9] in 20 ml. of ether under reflux for 3 hr. The solution was concentrated, leading to 0.57 g. of the ureide, **1-(3-carbethoxypropionyl)-1,3-bis(p-dimethylaminophenyl)urea**, m.p. 146–148°, from acetone–petroleum ether (b.p. 40–60°).

Anal. Calcd. for $C_{23}H_{30}N_4O_4$: C, 64.78; H, 7.09; N, 13.14. Found: C, 64.58; H, 7.21; N, 13.20.

Diethyl succinate (Eastman Kodak Co.) was distilled, b.p. 81° (6 mm.), n^{24}D 1.4184. This substrate, 0.315 g. (1.81 mmoles), was hydrolyzed at pH 7.2 by 0.050 g. of α-chymotrypsin as described above for diethyl malonate. Hydrolysis stopped after 4 hr., 1.78 ml. of 1 N sodium hydroxide being consumed, indicating 98% hydrolysis of one ester group. The solution was extracted, acidified, concentrated, and again extracted, as described for the malonate, leading to **ethyl hydrogen succinate**, 0.250 g. (1.72 mmoles), 97% yield. The infrared spectrum was identical with that of the authentic sample. This product was treated with 0.50 g. (1.6 mmoles) of 1,3-bis(p-dimethylaminophenyl)carbodiimide, as described above, leading to 0.52 g. (1.15 mmoles), 72% yield of the ureide, m.p. 147–148°, alone and when mixed with the authentic compound. The infrared spectra in Nujol were identical.

Diethyl glutarate (Eastman Kodak Co.) was distilled, b.p. 123 (11 mm.), n^{25}D 1.4238. Diethyl glutarate (0.694 g., 3.69 mmoles) was hydrolyzed at pH 7.2 by 0.050 g. of α-chymotrypsin, as described above. Hydrolysis was proceeding very slowly after 24 hr., 2.85 ml. of 1 N sodium hydroxide having been consumed, 77% hydrolysis of one ester group. The product was worked up in the usual way, leading to ethyl hydrogen glutarate in quantitative yield; its infrared spectrum was identical with those[2] of the synthetic sample and of the sample prepared by enzymatic hydrolysis at pH 7.8. The material was converted to the ureide, m.p. 157–158° alone and when mixed with the authentic sample[2]; the infrared spectra were identical.

Diethyl adipate (Eastman Kodak Co.) was distilled, b.p. 123° (8 mm.), n^{27}D 1.4245. This compound was subjected to the action of α-chymotrypsin under the conditions of the kinetic experiments below. The compound itself, 0.0216 g. (0.1 mmole), consumed 0.0001 ml./min. of 0.1 N sodium hydroxide, the enzyme alone consumed a like amount, and the compound and enzyme together consumed 0.0002 ml./min.

Diethyl α-acetamidomalonate (Matheson Coleman and Bell) was recrystallized from benzene–petroleum ether, m.p. 97–98°, lit.[20] m.p. 96°.

L-(−)-Diethyl N-Acetylglutamate.—L-Glutamic acid (Nutritional Biochemical Corp., 12 g. (0.082 mole)) was suspended in 100 ml. of absolute ethanol, and 16 g. of dry hydrogen chloride was added slowly, leading to a clear solution. The solution was concentrated *in vacuo* and the residue was dissolved in 100 ml. of chloroform. The solution was washed with cold saturated potassium carbonate, dried, saturated with hydrogen chloride, concentrated to one-third of its volume, and diluted with ether, leading to **L-(+)-diethyl glutamate hydrochloride**, 16 g. (0.067 mole), 82% yield, m.p. 113–114°, lit.[21] m.p. 114–115°, $[\alpha]^{21}$D +22.2° (c 3.51, ethanol). The ester hydrochloride, 15 g. (0.063 mole) was dissolved in 50 ml. of distilled water, neutralized with sodium acetate, and stirred with 35 ml. of acetic anhydride for 1 hr. at room temperature. The solution was concentrated and the residue was chromatographed on silica gel and eluted with chloroform–ethanol. The product[22] was distilled, 8.3 g. (0.034 mole), 51% yield, b.p. 154–156° (<1 mm.), n^{24}D 1.4580, $[\alpha]^{21}$D −20.3° (c 0.98, ethanol).

Anal. Calcd. for $C_{11}H_{19}NO_5$: C, 53.85; H, 7.75; N, 5.71. Found: C, 53.80; H, 7.64; N, 5.98.

(16) J. B. Jones and C. Niemann, *Biochemistry*, **2**, 498 (1963).
(17) G. E. Hein, J. B. Jones, and C. Niemann, *Biochim. Biophys. Acta*, **65**, 350 (1962).
(18) C. Contzen-Crowet, *Bull. Soc. Chim. Belges*, **35**, 183 (1926).
(19) E. Fourneau and S. Sabetay, *Bull. Soc. Chim. France*, [4], **43**, 861 (1928).
(20) L. Berlinguet, *Can. J. Chem.*, **33**, 1119 (1955).
(21) O. M. Friedman and A. M. Seligman, *J. Am. Chem. Soc.*, **76**, 658 (1954).
(22) E. Cherbuliez and Pl. Plattner, *Helv. Chem. Acta*, **12**, 321 (1929).

The infrared spectrum showed bands (μ): 3.05 (w), 3.4 (w), 5.7 (s), 6.0 (m), 6.5 (m), 6.9 (w), 7.3 (m), 7.8 (m), 8.3 (m), 8.8 (w), 9.1 (w), and 9.7 (m).

DL-**Diethyl N-acetylglutamate** was prepared similarly, 10 g. (0.068 mole) of DL-glutamic acid (Calbiochem, New York, N. Y.) leading to 6.3 g. (0.026 mole), 38% yield of the product, b.p. 163–165° (<1mm.), n^{25}D 1.4540. The infrared spectrum was identical with that of the L-compound.

Anal. Found: C, 53.8; H, 7.6.

D-(+)-**Diethyl N-acetylglutamate** was prepared similarly, 5.0 g. (0.034 mole) of D-glutamic acid (Calbiochem, New York, N. Y.) leading to 3.9 g. (0.016 mole), 48% yield, of **diethyl** D-**glutamate hydrochloride**, m.p. 113–115°, $[\alpha]^{21}$D −22.9° (*c* 2.56, ethanol) and thence to the product, 1.5 g. (0.0061 mole), 38% yield, m.p. 160–161° (<1 mm.), n^{27}D 1.4566, $[\alpha]^{21}$D +20.2° (*c* 2.94, ethanol). The infrared spectrum was identical with that of the L-compound.

Anal. Found: C, 53.8; H, 7.6; N, 6.0.

L-(+)-γ-**Ethyl-α-hydrogen N-Acetylglutamate.**—L-γ-Ethyl-α-hydrogen glutamate (3.0 g., 0.017 mole, Nutritional Biochemical Corp.) was dissolved in 5 ml. of water, neutralized with 1 *N* sodium hydroxide, boiled under reflux for 1 hr. with 15 ml. of acetic anhydride, and concentrated. The residue was extracted with 30 ml. of acetone and the extract was filtered and concentrated. This residue was dissolved in chloroform, chromatographed on silica gel, and eluted with 10% ethanol–chloroform, leading to an oil, b.p. 178–180° (0.4 mm.), n^{25}D 1.4660, $[\alpha]^{21}$D +4.67° (*c* 2.57, ethanol).

Anal. Calcd. for $C_9H_{15}NO_5$: C, 49.7; H, 6.91; N, 6.45. Found: C, 48.8; H, 6.91; N, 6.56.

The infrared spectrum, liquid film, showed bands (μ): 3.0 (m), 3.4 (m), 3.95 (w), 5.15 (w), 5.8 (s), 6.05 (s), 6.45 (s), 6.9 (m), 7.25 (s), 7.75 (s), 8.25 (s), 8.8 (m), 9.7 (m), 11.6 (w), and 12.7 (w).

L-(−)-**Diethyl N-acetylglutamate**, 0.66 g. (2.7 mmoles), was hydrolyzed at pH 7.2 by 0.020 g. of α-chymotrypsin, as described for diethyl malonate, 1.88 ml. of 1 *N* sodium hydroxide being consumed in 26 min., 70% hydrolysis of one ester group. (In another experiment, carried out for a longer time, hydrolysis stopped after 90% hydrolysis of one ester group.) The resulting solution was extracted, acidified, concentrated, extracted in the usual way, and chromatographed as described above, leading to L-(+)-γ-**ethyl-α-hydrogen N-acetylglutamate**, 0.40 g. (1.8 mmoles), $[\alpha]^{21}$D +4.90° (*c* 2.07, ethanol). Its infrared spectrum was identical with that of the synthesized sample. Attempts to prepare derivatives of this by treatment with 1,3-bis(*p*-dimethylaminophenyl)carbodiimide, with α-bromo-*p*-phenylacetophenone, and with *p*-toluidine failed.

DL-**Diethyl N-acetylglutamate**, 1.69 g. (6.9 mmoles), was hydrolyzed at pH 7.2 by 0.035 g. of α-chymotrypsin in the usual way, 3.47 ml. of 1 *N* sodium hydroxide being consumed in 2 hr., indicating 100% hydrolysis of one ester group of one enantiomorph. The solution was extracted with three 50-ml. portions of ether and the extract was dried and concentrated, leading to D-(+)-**diethyl N-acetylglutamate**, 0.70 g. (2.8 mmoles), 83% yield, $[\alpha]^{21}$D +19.5° (*c* 6.0, ethanol). Its infrared spectrum was identical with that of the synthesized sample. The aqueous reaction solution, after extraction, was brought to pH 3 with 5 *N* hydrochloric acid, concentrated, and extracted with three 30-ml. portions of acetone. The extract was dried and concentrated, leading to L-(+)-γ-**ethyl-α-hydrogen N-acetylglutamate**, 0.73 g. (3.3 mmoles), 97% yield, $[\alpha]^{21}$D +4.80° (*c* 7.3, ethanol). Its infrared spectrum was identical with that of the synthesized sample.

D-(+)-**Diethyl N-acetylglutamate**, 0.078 g. (0.32 mmole), was treated with 0.010 g. of α-chymotrypsin as described in the kinetic procedure; 0.1 ml. of 0.1 *N* NaOH was consumed in 25 min. and 0.017 ml. in the next 55 min., the reaction essentially stopping, 3.7% hydrolysis of one ester group.

α-Chymotrypsin was from Worthington Biochemical Corp., three times recrystallized, salt free. The molecular weight was assumed to be 25,000 in the calculations.

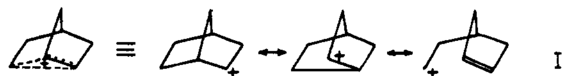
Kinetics of Hydrolysis by α-Chymotrypsin.—Reactions were carried out in the pH stat as previously described,[2] at pH 7.2, 25.0 ± 0.1°, in 20 ml. of 0.1 *M* sodium chloride, with magnetic stirring, under nitrogen. The nonenzymatic hydrolyses were followed for 20 min. and the needed corrections ascertained. In some cases the enzyme was added then as a solid, 0.010 g. dry weight, and the pH brought quickly back to 7.2. In other cases 5 ml. of a solution containing 0.010 g. of the enzyme in 0.1 *N* sodium chloride at pH 7.2 was added to 15 ml. of solution containing the substrate. Both methods led to the same results. The enzymatic hydrolysis was followed by the consumption of 0.1 *N* sodium hydroxide for 10–25 min., depending upon the reactivity of the substrate. Plots of alkali consumed due to enzymatic hydrolysis were linear with time. The results are summarized in Table I. The average corrections for nonenzymatic hydrolysis, referred to above, were for diethyl malonate 8%, succinate 1.4%, glutarate 4.3%, α-acetamidomalonate 1.8%, L-N-acetylglutamate 0.3%, and DL-N-acetylglutamate 0.6% of the enzymatic rates.

COMMUNICATIONS TO THE EDITOR

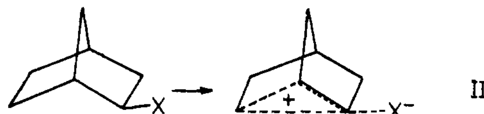
Rate of Solvolysis of 1-(*p*-Anisyl)camphene Hydrochloride. Evidence for the Absence of Significant Carbon Participation in the Solvolysis of a Tertiary Norbornyl Derivative

Sir:

The proposed nonclassical structure for the norbornyl cation distributes positive charge from the 2-position to the 1- and 6-carbon atoms[1] (I). Such delocalization



of the charge is presumed to stabilize the nonclassical structure over the classical and to stabilize the transition state with a partially formed nonclassical cation (II). It follows that it should be possible to explore the importance of such carbon participation in the transition state for the solvolysis of specific norbornyl



derivatives by determining the effect of alkyl or aryl substituents at the 1- and 6-positions on the rate of solvolysis. The well-known ability of such substituents to stabilize positively charged structures leads one to expect a marked rate-enhancing effect for such substituents *provided carbon participation is a significant factor in stabilizing the transition state for the norbornyl system under examination.*[2]

Both the n.m.r. spectra and the chemical data indicate that only one of the two anisyl groups stabilizes the 1,2-dianisylnorbornyl cation.[3] Consequently, it has been concluded that this ion must exist as a pair

(1) S. Winstein and D. Trifan, *J. Am. Chem. Soc.*, **74**, 1154 (1952).

(2) For a recent application of this approach to the bicyclo[3.1.0]hexane system, see E. J. Corey and H. Uda, *ibid.*, **85**, 1788 (1963).

(3) P. von R. Schleyer, D. C. Kleinfelter, and H. G. Richey, Jr., *ibid.*, **85**, 479 (1963).