Association of Substrates with α-Chymotrypsin, Diethyl α-Acetoxysuccinate, and Diethyl Malate[1]

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Abstract: Diethyl α-acetoxysuccinate and diethyl malate are hydrolyzed by α-chymotrypsin at the α-carbethoxyl group, leading to the β half-esters. The reactions are nonstereospecific: acetoxysuccinate, L, $K_{m,app}$ = 0.027 *M*, k_{cat} = 0.98 sec^{-1}; D, 0.012 *M*, 0.30 sec^{-1}; malate, L, 0.042 *M*, 2.7 sec^{-1}; D, 0.026 *M*, 1.7 sec^{-1}. Modes of association of substrates with enzyme are described and inversions of stereospecificity are explained. The β substituent is transoid to the hydrolyzing group; it is directed into the aryl (ar) site and may be aryl, alkyl, acyl, aroyl, or carbalkoxyl. The α-H is directed into the enzyme; this h position may be occupied by hydrogen, hydroxyl, or chlorine. The hydrolyzing group and α-acylamido group lie along the surface at the n and am sites, respectively. The am site may accommodate acetamido, hydroxyl, acyloxy, or carbalkoxymethylene groups. The β substituent increases reactivity by favorable effect on K_m and k_{cat}. The α-acetamido group increases reactivity by affecting k_{cat}, not K_m, affecting the entropy of activation of the hydrolytic reaction.

The associations of substrate and enzyme which result in specificity, the higher reactivity of one stereoisomer than another and of one compound than another, may be considered in terms of interactions of configurationally and conformationally oriented groups of substrates with complementarily located groups or sites of the enzyme.[2,3] The substrates and their substituents are of known structure, while the complementary groups of the enzyme are less well defined or quite unknown. Study of the enzymic reactions of substrates of varied, related structure may lead to information (i) about the size, geometry, and rigidity of the active site of the enzyme; (ii) about the structure of the substrate groups which may associate at each part of the active site and, so, about the nature of the several parts of the active site; and (iii) about structural requirements in the substrate for reactivity, stereospecificity, and inversion of stereospecificity.

In hydrolyses catalyzed by α-chymotrypsin, esters and amides of N-acyl-β-phenylalanine, $RCONHCH(CH_2C_6H_5)COX$, and other N-acyl-β-arylalanines are very reactive and these compounds are related to the site of attack by α-chymotrypsin on proteins.[4] Compounds related to these substrates may be used to set up a model in outline of the space in the enzyme in which they fit during reaction, and on the surface of which are located the groups with which the substrate groups associate and/or react. The key substituents attached to the two tetrahedral carbon atoms of ethyl L-N-acetylphenylalaninate are the β-phenyl group, the α-acetamido group, the α-carbethoxyl group, and the α-hydrogen atom; the complementary areas on the active site of the enzyme may be referred to as the aryl, ar, acetamido, am, carbethoxy or nucleophilic, n, and hydrogen, h, sites, respectively. A possible conformation of this substrate in its association with an enzyme is indicated in Figure 1.

The aryl group and its site ar are of particular importance in reactions of α-chymotrypsin, leading to the very favorable binding of substrate to enzyme which is necessary but not sufficient for high reactivity. This binding is such that all uncharged aromatic compounds which have been examined, including simple hydrocarbons and alcohols, and D enantiomorphs of reactive L compounds, associate well with α-chymotrypsin and act as competitive inhibitors.[5-7] In small molecule substrates and in proteins these aryl groups are side chains which may fit into the enzyme, and the site ar may be a hole or fold into the interior in which the aryl or related groups may fit.[8] Aliphatic groups are also bound well, indicating that this association is essentially nonpolar.[9-11]

In initial studies of variations in structural features, the symmetric compounds, diethyl α-acetamidomalonate,[12] $CH_3CONHCH(CO_2C_2H_5)_2$, and diethyl β-acetamidoglutarate,[13] $CH_3CONHCH(CH_2CO_2C_2H_5)_2$, were hydrolyzed slowly and stereospecifically, probably in the L sense, by α-chymotrypsin. Even in the absence of the β-aryl group which is characteristic of natural substrates for this enzyme, it distinguished between the two chemically equivalent, enantiomorphic carbethoxyl groups in each of these substrates. The optically active half-acids were formed. This may be accounted for, in conformational terms, by classical diastereomeric interactions between one symmetric tetrahedral grouping of type Cabdd and a second dissymmetric tetrahedral grouping. But, in addition, although the glutarate was only slowly hydrolyzed by α-chymotrypsin, it was far more reactive than the related com-

(1) We are pleased to acknowledge generous support by the Division of Research Grants, National Institutes of Health, GM-04584, and a Fellowship under Title IV of the National Defense Education Act (S. Y. W.). This is paper XII on specificity of reactions of α-chymotrypsin.

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

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

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

(5) R. A. Wallace, A. N. Kurtz, and C. Niemann, *Biochemistry*, **2**, 824 (1963).

(6) R. J. Foster, H. J. Shine, and C. Niemann, *J. Am. Chem. Soc.*, **77**, 2378 (1955).

(7) R. J. Foster and C. Niemann, *ibid.*, **77**, 3370 (1955).

(8) M. L. Bender, J. V. Killheffer, Jr., and F. J. Kézdy, *ibid.*, **86**, 5331 (1964).

(9) R. Jennings and C. Niemann, *ibid.*, **75**, 4687 (1953).

(10) J. B. Jones and C. Niemann, *Biochemistry*, **2**, 498 (1963).

(11) G. E. Hein, J. B. Jones, and C. Niemann, *Biochim. Biophys. Acta*, **65**, 350 (1962).

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

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

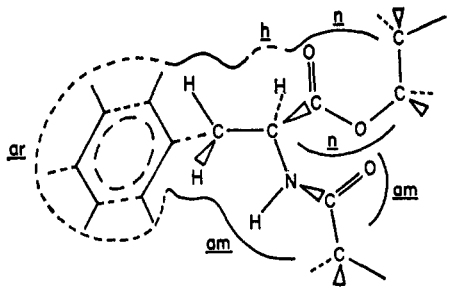


Figure 1. Association of ethyl L-N-acetyl-β-phenylalaninate with α-chymotrypsin.

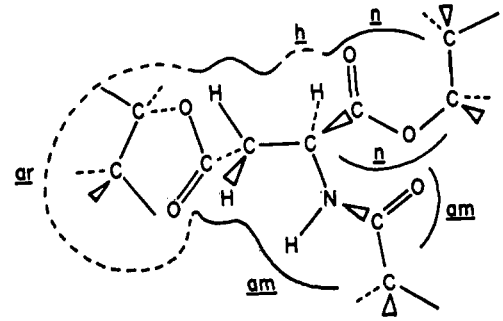


Figure 2. Association of diethyl L-N-acetylaspartate with α-chymotrypsin.

pound which lacked the second carbethoxyl group, ethyl β-acetamidobutyrate,[14] $CH_3CONHCH(CH_3)CH_2CO_2C_2H_5$, which was not hydrolyzed at all by the enzyme. One carbethoxyl group in the glutarate was essential for the stereospecific hydrolysis of the other carbethoxyl by the enzyme. It seemed likely that it had this effect by acting like a β-aryl group and associating at the ar site in the enzyme.

Diethyl L-N-acetylaspartate, $CH_3CONHCH(CH_2CO_2C_2H_5)CO_2C_2H_5$, would then be a far better substrate for α-chymotrypsin, with the group $-CO_2C_2H_5$ taking the place of the phenyl group, $-C_6H_5$, and the other three substituents fitting in normally as indicated in Figure 2. This was borne out in fact;[15] the aspartate was hydrolyzed highly stereospecifically and rapidly by α-chymotrypsin: $K_m = 0.023\ M$, $k_{cat} = 22\ sec^{-1}$. This indicated that derivatives of esters of dibasic acids might provide available, water-soluble substrates with which to explore the active site. The diethyl esters of the unsubstituted dibasic acids, diethyl malonate through diethyl adipate, were examined with α-chymotrypsin.[16] Diethyl succinate showed most favorable $K_{m,app}$ and k_{cat}, and was comparable in reactivity to the phenyl analog, ethyl β-phenylpropionate. Diethyl malonate and diethyl glutarate were less readily hydrolyzed, while diethyl adipate was inert. This indicated that the distance between the ar and n sites in the enzyme may be spanned by one methylene group, is best bridged by two methylenes, and can accommodate three methylene groups but not four. While the substrates will adopt their most favorable conformations, some distortion in the active site may be possible.

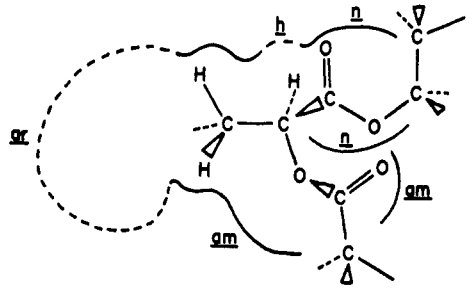
The diethyl esters of the first three homologous α-acetamido dibasic acids were found to hydrolyze with L specificity, 20, 130, and 40 times more rapidly than the corresponding unsubstituted diesters. They were also far more reactive than ethyl L-N-acetylalaninate,[17] which lacks the second carbethoxyl group.

The α-acetamido group in these substrates probably associates with the enzyme by hydrogen-bonding interactions. The amide hydrogen of the acetamido group appears essential for its normal function since (i) N-methylation of the aspartate derivative prevents its hydrolysis by α-chymotrypsin,[15] and (ii) replacement of the acetamido by acetoxyl, CH_3CONH- by CH_3COO-, leads to entirely different effects. In the simplest example, while ethyl and methyl L-N-acetylalaninate, $CH_3CONHCH(CH_3)CO_2R$, were poorly bound and hydrolyzed stereospecifically,[3,17] ethyl L-α-acetoxypropionate, $CH_3COOCH(CH_3)CO_2C_2H_5$, appeared to be bound well but was essentially inert,[18] while the D enantiomorph was hydrolyzed.[3,18] From this inversion of stereospecificity it was inferred that, while the acetoxy group, $-OOCCH_3$, may interact with the enzyme at the am site (Figure 3A), in the absence of a β substituent it prefers to associate like a carbethoxymethylene, $-CH_2COOC_2H_5$, or like a benzyl group, $-CH_2C_6H_5$, *i.e.*, in effect it provides a β substituent to the ar site, the alkyl oxygen replacing the β-methylene group. In ethyl L-α-acetoxypropionate (Figure 3B), this does not leave the carbethoxy group at the nucleophilic site, while in the D enantiomorph it does (Figure 3C), and the latter was hydrolyzed.

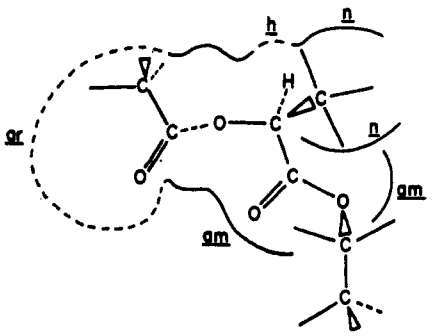
However, in ethyl β-phenyl-α-acetoxypropionate[19] with both a β-phenyl and an α-acetoxy group present, the β-aryl group strongly preferred to associate at its normal ar site, the acetoxyl group was placed at am, and high L specificity resulted. The reactivity was greatly reduced as compared with the β-phenyl-α-acetamido compound.[10] On the other hand diethyl β-acetoxyglutarate, $CH_3COOCH(CH_2CO_2C_2H_5)_2$, was more reactive than the corresponding β-acetamidoglutarate[20] and showed no stereospecificity,[3] while the slower hydrolysis of the β-acetamidoglutarate was stereospecific, apparently in the L sense.[13] In the β-acetamidoglutarate, the second carbethoxyl group, the β-acetamido group, and the β-hydrogen may bind to the enzyme quite like the β-carbethoxyl, α-acetamido, and α-hydrogen in the aspartate (Figure 2), and lead to L stereospecificity. This association places the reacting carbethoxyl rather firmly one methylene removed from the complex nucleophilic site; a distortion is required for reaction, and low reactivity results.

In the β-acetoxyglutarate the hydrogen-bonding interaction is not present, and, as in Figure 3, the acetoxyl group may associate at either the am or the ar sites. When it is at the am site (Figure 4A), the second carbethoxy group, $-CO_2R'$, is at the ar site and hydrolysis of $-CO_2R$ occurs in the L sense. When the acetoxyl group is at the ar site (Figure 4B), the first carbethoxymethylene group, $-CH_2CO_2R$, may be at the am site and hydrolysis may occur in the D sense. The relatively rapid nonstereospecific hydrolysis of diethyl β-acetoxyglutarate indicates that alkoxymeth-

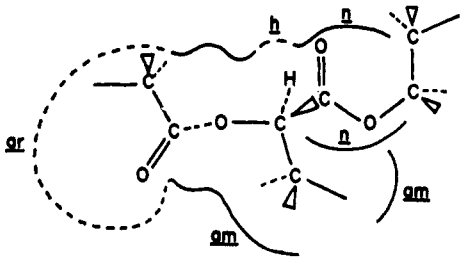
(14) S. G. Cohen, Y. Sprinzak, and E. Khedouri, *J. Am. Chem. Soc.*, **83**, 4225 (1961).
(15) S. G. Cohen, J. Crossley, and E. Khedouri, *Biochemistry*, **2**, 820 (1963).
(16) S. G. Cohen and J. Crossley, *J. Am. Chem. Soc.*, **86**, 4999 (1964).
(17) J. P. Wolf, III, and C. Niemann, *Biochemistry*, **2**, 493 (1963).
(18) S. G. Cohen, J. Crossley, E. Khedouri, and R. Zand, *J. Am. Chem. Soc.*, **84**, 4163 (1962).
(19) S. G. Cohen and S. Y. Weinstein, *ibid.*, **86**, 5326 (1964).
(20) S. G. Cohen and J. Crossley, *ibid.*, **86**, 1217 (1964).



A



B



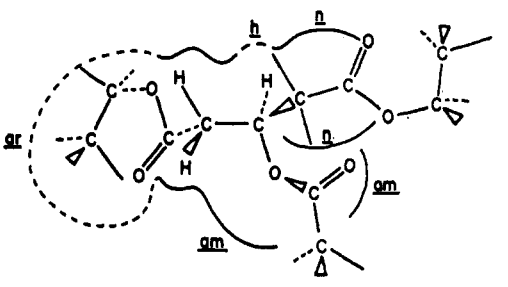
C

Figure 3. Associations of: L-and D-ethyl α-acetoxypropionate with α-chymotrypsin: A, ethyl L-α-acetoxypropionate, orientation not preferred; B, ethyl L-α-acetoxypropionate, orientation not reactive; C, ethyl D-α-acetoxypropionate, orientation reactive.

ylene, $-CH_2COOR$, and acetoxyl, $-OOCCH_3$, are similar to each other and may have equivalent tendencies for associations at the ar and am sites. Their polar properties are fairly similar, and their steric requirements are such that they may each fit into the ar and am sites. In the absence of the hydrogen-bonding interactions, rigidity is decreased and the system more readily accommodates the distortion required to fit three methylene groups between sites ar and n. This leads to more favorable K_m and k_{cat} in the β-acetoxy- than in the β-acetamidoglutarate. The equivalence of the acetoxyl and carbethoxymethylene groups was unexpected and merited further study in diethyl α-acetoxysuccinate, which will be described below.

While association of the α-acetamido group at the am site may involve hydrogen bonding, the α-hydroxyl is far less effective in leading to reactivity and stereospecificity. Ethyl lactate is hydrolyzed without stereospecificity[21,22] and its kinetic constant

(21) S. G. Cohen and E. Khedouri, *J. Am. Chem. Soc.*, **83**, 4228 (1961).



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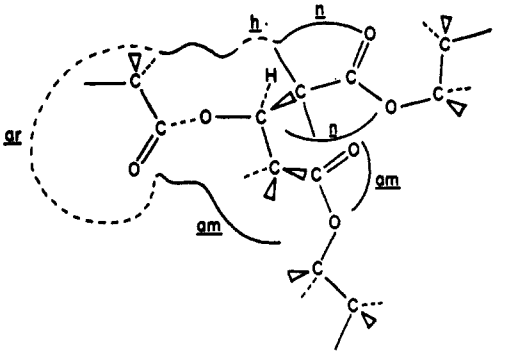


B

Figure 4. Associations of diethyl β-acetoxyglutarate with α-chymotrypsin: A, L sense; B, D sense.

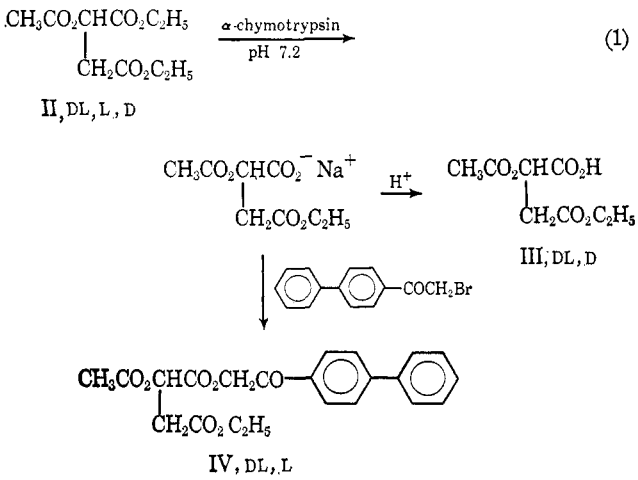
k_{cat} is much smaller than that for the stereospecific hydrolysis of methyl N-acetylalaninate.[17] Similarly, diethyl α-hydroxymalonate appeared to be hydrolyzed without stereospecificity.[21,23] In the derivatives of ethyl β-phenylpropionate, the α-hydroxy compound hydrolyzes with partial stereospecificity,[19,24] while the α-acetamido compound is much more reactive and highly stereospecific. From these results it appears that while the α-hydroxyl group may fit into the am position, leading to hydrolysis in the L sense, it may be small enough to fit into the h site also, leading to hydrolysis in the D sense and to diminished or no stereospecificity. This inference with respect to the α-hydroxyl group merited further consideration, as in the case of malate esters, which will be described below.

Results

The diethyl malates I, L-(−), D-(+), and DL, were prepared by treatment of the corresponding malic acids with ethanol and hydrochloric acid. The diethyl α-acetoxysuccinates II, L-(−), D-(+), and DL, were prepared by treatment of the malates with acetic anhydride in pyridine.

DL-Diethyl α-acetoxysuccinate was hydrolyzed readily by α-chymotrypsin. Hydrolysis of 0.66 g (2.8 mmoles) of the ester at pH 7.2 by 0.074 g of α-chymotrypsin was complete in 2 hr for hydrolysis of one ester group, the reaction then stopping (eq 1).

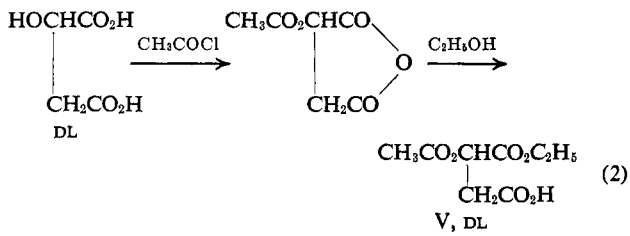
(22) I. Tinoco, *Arch. Biochem. Biophys.*, **76**, 148 (1958).
(23) S. G. Cohen, R. Schultz, and S. Y. Weinstein, *J. Am. Chem. Soc.*, **88**, 5313 (1966).
(24) J. E. Snoke and H. Neurath, *Arch. Biochem. Biophys.*, **21**, 351 (1949).



This reaction did not involve hydrolysis of the acetoxyl group to form the malate diester, which would have hydrolyzed further, as described below. The reaction led to a high yield of optically inactive β-ethyl hydrogen α-acetoxysuccinate (III), mp 71–72° (eq 1).

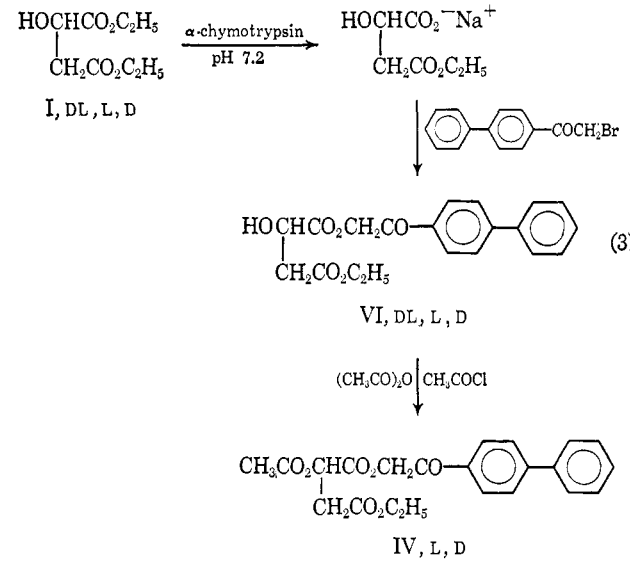
In another experiment, the sodium salt of acid III was converted directly in good yield to optically inactive α-(*p*-phenylphenacyl) β-ethyl α-acetoxysuccinate (IV) (eq 1). Consumption of alkali during the hydrolysis was uniform, showing no appreciable break after consumption of 1 equiv of alkali for one ester group of one enantiomorph. The hydrolysis appeared substantially nonstereospecific.

Assignment of the structures of III and IV, based on hydrolysis of the α ester and not the β, seems secure. The α-acetoxyl group is electron attracting and would be expected to increase the base-catalyzed hydrolysis of the α-carbethoxyl group as compared with the β-. However attempts to prepare III by treatment of II with 1 equiv and with 0.5 equiv of dilute base failed. The three ester groups of II were apparently not sufficiently different in reactivity toward hydroxide, while the enzymatic reaction was specific. Treatment of DL-malic acid with acetyl chloride led to DL-α-acetoxysuccinic anhydride, and reaction of this with ethanol, in which nucleophilic attack occurs at the more activated α-carbonyl (eq 2), led to the previously reported[25] isomer of III, DL-α-ethyl hydrogen α-acetoxysuccinate (V). The melting point of a mixture of III and V was depressed, confirming the structure of III.



Hydrolysis of L-(−)- and D-(+)-diethyl α-acetoxysuccinate by α-chymotrypsin at pH 7.2 led to similar results. Hydrolysis of 0.62 g (2.7 mmoles) of the L compound by 0.066 g of the enzyme was complete for one ester group in 80 min and led to recovery in 72% yield of L-(−)-α-(*p*-phenylphenacyl) β-ethyl α-acetoxysuccinate (L-IV, eq 1). Hydrolysis of 0.58 g (2.5 mmoles) of the D ester by 0.083 g of the enzyme was complete for one ester group in 90 min and led to recovery in 88% yield of D-(+)-β-ethyl hydrogen α-acetoxysuccinate (D-III, eq 1).

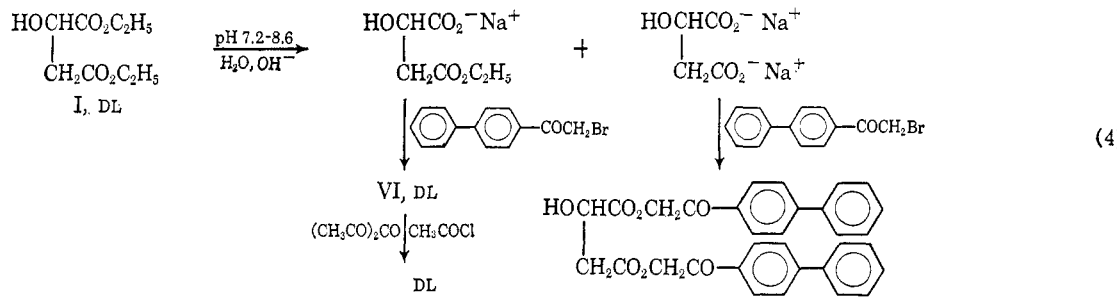
DL-Diethyl malate was also hydrolyzed readily with little stereospecificity by α-chymotrypsin. Hydrolysis of 0.91 g (4.8 mmoles) of the ester at pH 7.2 by 0.085 g of the enzyme was 86% complete for one ester group in 80 min. Attempts to isolate the monoester monoacid as a crystalline solid were unsuccessful, but treatment of the sodium salt, obtained by dialysis, with *p*-phenylphenacyl bromide led to DL-α-(*p*-phenylphenacyl) β-ethyl malate (VI, eq 3).



Hydrolysis of L-(−)- and D-(+)-diethyl malate separately by α-chymotrypsin proceeded well at pH 7.2. Hydrolysis of 0.64 g (3.4 mmoles) of the L compound by 0.052 g of the enzyme was 94% complete after 1 hr, and from the product the L-(+)-α-(*p*-phenylphenacyl) β-ethyl malate (VI) was obtained in 22% yield. This was characterized by conversion to L-(−)-α-(*p*-phenylphenacyl) β-ethyl α-acetoxysuccinate (IV), identical with that prepared *via* enzymic hydrolysis of L-(−)-diethyl α-acetoxysuccinate (eq 3). Similarly, D-(+)-diethyl malate, 0.72 g (3.8 mmoles), was hydrolyzed by 0.060 g of α-chymotrypsin, 95% of one ester group hydrolyzing in 2 hr. The product led to D-(−)-α-(*p*-phenylphenacyl)-β-ethyl malate and this was converted by acetylation to the D-(+)-acetoxy compound.

Assignment of the structures of these compounds as arising from enzymic hydrolysis of the α-ester group was further indicated by comparison with the product of hydrolysis of DL-diethyl malate, at pH 7.2–8.6 (eq 4). The reaction was slow, the equivalent of one ester group of 4 mmoles of the diester being hydrolyzed in 2 days. Treatment of the product mixture with *p*-phenylphenacyl bromide led to the derivative of the product of hydrolysis of both ester groups, DL-di-*p*-phenylphenacyl malate, and to that of hydrolysis of the α-ester group, DL-α-(*p*-phenylphenacyl) β-ethyl malate. The latter was identical with DL-VI obtained *via* the enzymic hydrolysis of DL-diethyl malate (eq 3) and was characterized further by acetylation, leading to a product identical with DL-IV (eq 4).

(25) D. H. Horn and Y. Y. Pretorius, *J. Chem. Soc.*, 1462 (1954).



(4)

The kinetics of hydrolysis of L-(−)-, D-(+)-, and DL-α-acetoxysuccinate by 0.1 mg/ml of α-chymotrypsin and of L-(−)-, D-(+)-, and DL-diethyl malate by 0.04 mg of α-chymotrypsin/ml were studied. Zero-order initial rates of hydrolyses of varying initial concentrations of these esters are given in Tables I and II. Double reciprocal plots of the data for the D and L compounds were linear. Values of the kinetic parameters $K_{m,app}$ and k_{cat} were calculated by the least-squares procedure and are given in Table III. Included for comparison are data for diethyl N-acetylaspartate, diethyl succinate, methyl β-phenylpropionate, and ethyl β-phenyl-α-acetoxypropionate.

Table I. Hydrolysis of Diethyl α-Acetoxysuccinate by α-Chymotrypsin, 0.1 mg/ml, 0.095 *M* NaCl, pH 7.2, 25°

[S] × 10^3, *M*	$V_E \times 10^7$, *M* sec^{-1}
L-(−) 3.45	4.0
4.29	4.8
5.18	5.9
6.17	6.5
7.92	8.0
8.49	8.4
D-(+) 4.16	2.8
5.00	3.3
5.71	3.4
6.24	3.8
7.45	4.2
DL 4.96	4.2
5.79	4.8
6.77	5.6
7.60	6.2
8.00	6.5

Table II. Hydrolysis of Diethyl Malate by α-Chymotrypsin, 0.04 mg/ml, 0.095 *M* NaCl, pH 7.2, 25°

[S] × 10^3, *M*	$V_E \times 10^7$, *M* sec^{-1}
L-(−) 5.05	4.3
5.93	4.8
6.94	5.6
8.58	6.5
10.95	7.6
12.80	9.5
14.16	10.1
D-(+) 6.13	4.4
6.92	5.4
8.48	5.9
9.60	6.5
10.6	6.6
14.3	9.1
DL 7.58	5.1
10.4	6.5
14.6	8.7

Table III. Kinetic Constants for Hydrolysis of D- and L-Diethyl α-Acetoxysuccinate and D- and L-Diethyl Malate, 0.095 *M* NaCl, pH 7.2, 25°

	$K_{m,app}$, *M*	k_{cat}, sec^{-1}	$k_{cat}/K_{m,app}$	Ref
L-(−)-Diethyl α-acetoxysuccinate	0.027	0.98	36	..
D-(+)-Diethyl α-acetoxysuccinate	0.012	0.30	25	..
L-(−)-Diethyl malate[a]	0.042	2.7	64	..
D-(+)-Diethyl malate	0.026	1.7	65	..
L-(−)-Diethyl N-acetylaspartate[b]	0.023	22	950	15
Diethyl succinate[b]	0.0087	0.065	7.5	16
Methyl β-phenylpropionate[c]	0.004	0.03	7.5	*d*
L-Ethyl α-acetoxy-β-phenylpropionate[e]	0.023	0.6	26	19

[a] J. P. Wolf, III, and C. Niemann, *Biochemistry*, **2**, 493 (1963), reported that the α-chymotrypsin-catalyzed hydrolysis of L-dimethyl malate was first order in substrate, $k_0/K_m = 12$, pH 7.9, 0.5 *M* NaCl. [b] 0.1 *M* NaCl, pH 7.2. [c] 20% methanol-water, 0.1 *M* NaCl, pH 7.8 phosphate–borate buffer. [d] K. J. Laidler and M. L. Barnard, *Trans. Faraday Soc.*, **52**, 497 (1956). [e] 20% ethanol-water, 0.055 *M* NaCl, pH 7.8.

Discussion

The unsubstituted parent compound diethyl succinate, with low $K_{m,app}$, appears to be bound better than its α-substituted derivatives but has low k_{cat} and low reactivity. Its reactivity is similar to that of the phenyl analog, methyl β-phenylpropionate, and the second carbethoxy group presumably binds at the ar site as indicated in Figure 2.

The α-acetoxysuccinate (Table III) showed increased reactivity both for the L and D forms as compared with the parent succinate. The kinetic constants K_m and k_{cat} differed by a factor of 2–3 for the two enantiomorphs, but the reactivities, k_{cat}/K_m, were similar, being only 50% greater for the L than for the D compound, and the reactions were essentially nonstereospecific. This is consistent with the inference drawn from the relatively rapid nonstereospecific hydrolysis of diethyl β-acetoxyglutarate, that the acetoxy and carbethoxymethylene groups, $-OOCCH_3$ and $-CH_2COOC_2H_5$, are similar in their polar properties and steric requirements. The L- and D-diethyl α-acetoxysuccinates may associate with the enzyme and react as indicated in Figure 5A and B. The L compound associates like ethyl L-aspartate and ethyl L-N-acetyl-β-phenylalaninate (Figures 1 and 2); the D compound has the carbethoxymethylene group at the am site, the acetoxy group at ar, placing the hydrolyzing ester group at n. The binding, as indicated by $K_{m,ap}$, is similar to that of the derivative of L-aspartate, but values of k_{cat} and over-all activity were far less for both compounds than for the aspartate. While the acetoxy and carbethoxymethylene groups may fit in the am site, they lack the NH group and do not

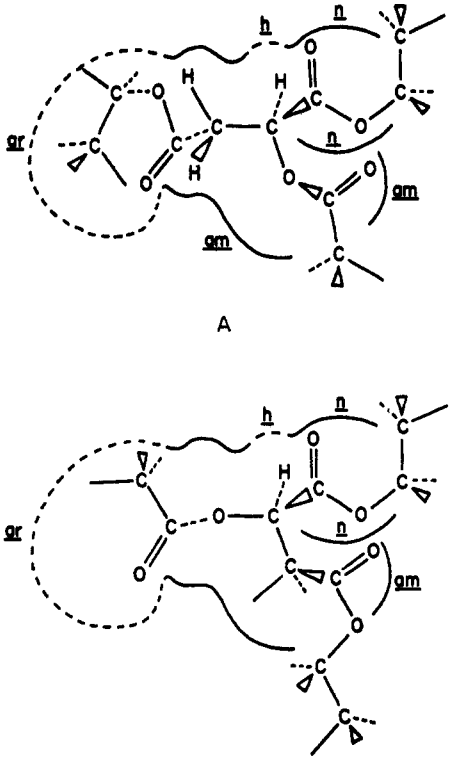


Figure 5. Associations of L- and D-diethyl α-acetoxysuccinate: A, L-diethyl α-acetoxysuccinate; B, D-diethyl α-acetoxysuccinate.

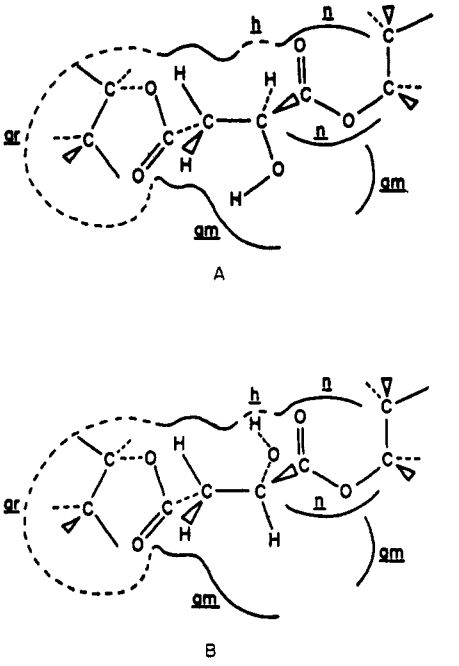


Figure 6. Associations of L- and D-diethyl malate: A, L-diethyl malate; B, D-diethyl malate.

hydrogen bond effectively, and they may not organize the enzyme–substrate complex adequately for high reactivity to result. The increased reactivity over the unsubstituted succinate may reflect in part the higher reactivity toward nucleophilic reaction due to the electron-attracting α-acetoxyl substituent.

Ethyl L-α-acetoxy-β-phenylpropionate (Table III) showed reactivity very similar to that of the L- and D-α-acetoxysuccinates. This reaction was stereospecific, the D compound being inert. The L compound associated with the enzyme as in Figure 5A. While the acetoxy and carbethoxymethylene groups may be almost equivalent in their associations at ar and am, the benzyl group strongly prefers the ar site and prefers not to associate at the am site. The am site prefers to accommodate an acyclic chain connected to the α-carbon by an unbranched group, –NH– (Figures 1 and 2), –O– (Figures 4A, 5A), and $-CH_2$ (Figures 4B, 5B).

The association of the α-acetamido group with the enzyme may involve rather firm hydrogen bonding, with the –NHCO– group both donating and accepting a proton. The acetamido group as a result strongly prefers to associate at the am site and leads to high stereospecificity.

The α-hydroxyl substituent may also form a hydrogen bond at the am site, but less effectively than the amide. The diethyl malates (Table III) were more reactive than the parent succinate by an order of magnitude; they had less favorable $K_{m,app}$, far more favorable k_{cat}. The L and D compounds showed very similar $K_{m,app}$ and k_{cat}, and identical $k_{cat}/K_{m,app}$ ratios, consistent with the nonstereospecific hydrolyses of ethyl lactate[21,22] and diethyl α-hydroxymalonate[21] and the partial stereospecificity in the hydrolysis of ethyl α-hydroxy-β-phenylpropionate.[19,24] It seems that in all these reactions the α-hydroxyl may be at the am site for hydrolysis of the L enantiomorph and, being small, it may readily be at the h site for hydrolysis of the D, as in Figure 6A,B. That a small group other than hydrogen may be at the h site is also indicated in the nonstereospecific hydrolysis of the ethyl α-chloro-β-phenylpropionate.[24]

A remarkable example of stereospecificity due to the hydroxyl group is that observed in the hydrolysis by α-chymotrypsin of diethyl β-hydroxyglutarate.[21] In the absence of all structural features of the natural substrates, without aryl and acylamido groups, with excess length between the binding (ar) and hydrolyzing (n) ester groups, this symmetric molecule of type Cabdd was hydrolyzed slowly but with high stereospecificity in the L sense. Enzymic reaction depends upon prior association. It may be that, unlike the succinates, because of the excessive chain length, the glutarates do not provide effective association by the two carbethoxyl groups at the ar and n sites. The only two groups properly placed for association are the second carbethoxyl group at ar and the hydroxyl at am, leading to L stereospecificity. As in the β-acetamidoglutarate, distortion is required to accommodate the α-methylene group and to place the hydrolyzing ester group at the n site, and low reactivity is observed. In the malates the two ester groups provide adequate association, and the hydroxyl goes to am and to h in the associations of L and D compounds, respectively.

The reacting conformation of substrates depicted in Figures 1–6 is similar to that of Hein and Niemann.[2] It has the substituents on the α- and β-carbon atoms staggered and the hydrolyzing ester or amide group transoid to the β-aryl or β-carbethoxyl group. Support for this conformation is seen in the effective hydrolysis

of diethyl fumarate by α-chymotrypsin and the inertness of diethyl maleate.[26] The β substituent is indicated as pointing into the enzyme, and it is largely responsible for effective binding. It is preferably linked to the α-carbon by a group that is unbranched or not further substituted, by $-CH_2-$, $-O-$,[18] or even $-NH-$.[27] The β substituent may be aryl, alkyl,[11] acyl,[18] aroyl,[19] or carbalkoxy.[15]

The α-hydrogen appears to be necessary for high reactivity since compounds with a quaternary α-carbon are comparatively unreactive.[14,28] It seems improbable that the α-hydrogen contributes a strong positive interaction at its site h, and likely that this effect is steric. The other three groups on the α-carbon might align themselves so that the fourth group, H, would be directed away from the enzyme surface and be both sterically and electronically innocuous. This does not appear to be the case. It appears that proper location of the other three groups on the enzyme directs the hydrogen into the enzyme, in a region of restricted volume. A hydroxyl or chlorine may be accommodated but a substantially larger group may not.

The ester or amide group which is to be hydrolyzed by the endopeptidase represents a segment of a protein substrate and probably makes contact with the proton-transferring imidazole group and nucleophilic serine hydroxyl at an exposed or surface region of the enzyme along which a portion of a polypeptide might lie. The acylamido group in small molecule substrates also represents a segment of a protein substrate and may associate at an exposed region along which a part of the N-terminal portion of a polypeptide could lie.

The acyl group is in nature that of an aliphatic amino acid. When aromatic acyl groups are used in synthetic substrates the aroylamido group shows increased tendency to associate at the ar site. This results in increased capacity of methyl D-N-benzoylalaninate[29] to be hydrolyzed by α-chymotrypsin, and decreased stereospecificity as compared with the N-acetylalaninate. The D-N-benzoylalaninate showed essentially the same $K_{m,app}$ and k_{cat} as ethyl β-phenylpropionate and may associate with the enzyme as indicated in Figure 3C for ethyl D-α-acetoxypropionate. The preferred orientation is the normal one and methyl L-N-benzoylalaninate was hydrolyzed more rapidly than the D.[29] However, inversion of stereospecificity was observed in methyl N-picolinylalaninate and may be accounted for in this way. The increased tendency of an α-aroyl group to associate at the ar site is seen in ethyl α-benzoyloxypropionate which showed marked D specificity.[19] The D enantiomorph associates as indicated in Figure 3C, and $K_{m,app}$ and k_{cat} were very similar to those of methyl β-phenylpropionate and methyl D-N-benzoylalaninate.

The am site accommodates, in small molecule substrates, groups which have steric requirements less than or similar to that of the acylamido group, RCONH–. These are hydroxyl, the acyloxy group, RCOO–, and the carbalkoxymethylene group, ROOCCH_2–. Association of the latter two groups at am may suffice to lead to stereospecificity and to some increase in reactivity. The α-acetamido group increases reactivity markedly, by leading to higher values of k_{cat} and not by improved binding, as indicated by values of K_m. That this important effect is not accompanied by more favorable free energy for the over-all association than would be provided by association at the ar site alone implies that the enthalpy of association by the acetamido group at the am site may be balanced or outweighed by the entropy, and this effect is basic in the enzymic catalysis. The nonenzymic hydrolysis of esters is a reaction of low activation enthalpy,[30] ~12 kcal mole^{-1}, and proceeds slowly at neutrality because of very unfavorable entropy[30] of activation, ~−27 cal degree^{-1} mole^{-1}. The hydrogen-bonding interactions of the α-acetamido group with the enzyme may lead to organization of the complex such that thereby degrees of freedom are lost by the substrate and by groups on the enzyme. The imidazole and serine hydroxyl of the n site of the enzyme and the carbethoxyl of the substrate may be placed and held in position favorable for reaction, increasing k_{cat} by making more favorable the entropy of activation.[31,32] Association of aryl or carbethoxyl at the ar site also contributes to the rigidity of this organization and to increased values of k_{cat}. The activation enthalpy for the hydrolysis of amides is somewhat higher[33] than for esters, and amides are less reactive both in nonenzymic and enzymic hydrolyses. The entropy of activation for hydrolysis of amides is unfavorable,[34] ~−20 cal deg^{-1} mole^{-1}, and the enzyme may act on amides also in large part by affecting the entropy of activation.[35]

Finally, the D specificity found in hydrolysis of the cyclic substrate 1-keto-3-carbomethoxytetrahydroisoquinoline[36] may perhaps be accounted for in accord with the preceding considerations. This substrate is a condensed cyclized analog of methyl-N-benzoyl-β-phenylalaninate. The tendency of α-aroylamido and α-aroyloxy groups to increase D reactivity by having the α substituent associate at the β-aryl site has been noted. In this cyclic substrate the one aromatic ring takes the place of both the β substituent and the N-aroyl group, and its action as the β substituent dominates. The configurations and conformations of esters of L- and D-N-acyl-β-phenylalaninate and of L- and D-1-keto-3-carbomethoxytetrahydroisoquinolines and their possible associations with the enzyme (Figure 7) lead to the following considerations.

The D-β-phenylalaninate will not react since, in placing the aryl and carbalkoxy groups in the transoid conformation and in their respective sites, this molecule would require the large acylamido group to be

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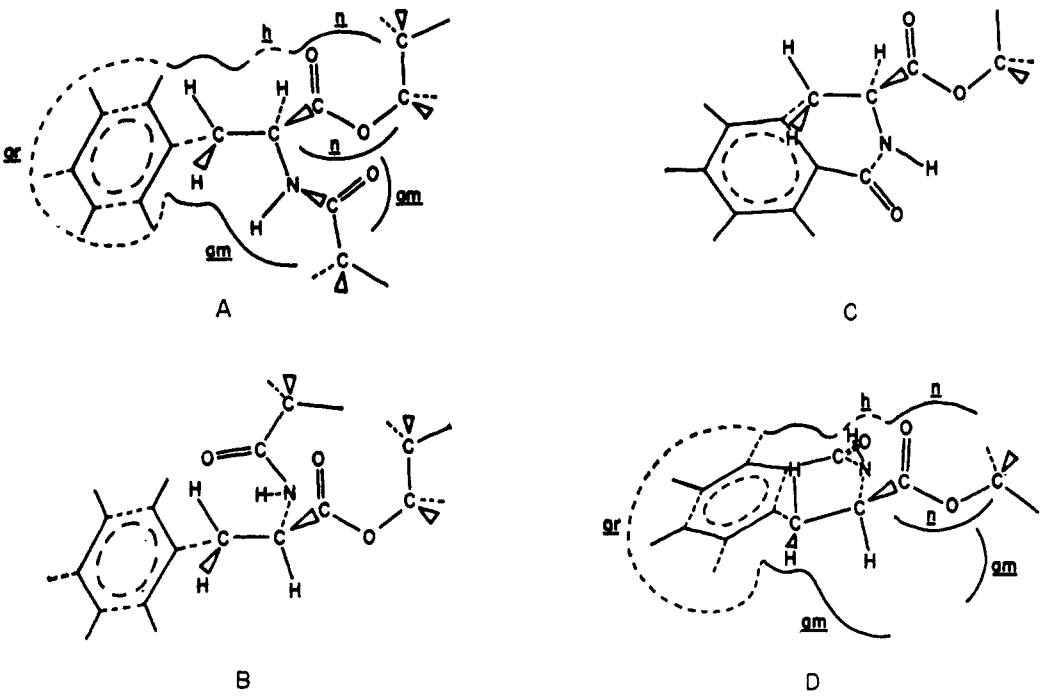

Figure 7. Comparison of derivatives of phenylalanine and of tetrahydroisoquinoline: A, ethyl L-N-acetyl-β-phenylalaninate; B, ethyl D-N-acetyl-β-phenylalaninate; C, L-1-keto-3-carbomethoxytetrahydroisoquinoline; D, D-1-keto-3-carbomethoxytetrahydroisoquinoline.

directed into the enzyme, into the restricted h site. In the L enantiomorph the acylamido group lies along the surface, the α-hydrogen pointing inward. However, in the D-1-keto-3-carbomethoxytetrahydroisoquinoline, the acylamido group, directed inward, is condensed with the similarly directed aryl group; the relatively small NH may be in the h site and the carbonyl is incorporated into the aryl group which remains directed into the enzyme and is displaced only little from the ar site. The L-tetrahydroisoquinoline may not place the NH in the h site without removing the carbalkoxy group from the n site and preventing reaction. When the NH group remains at the am site if the ester is to have a possibility of hydrolyzing, the condensation with the aryl group draws the latter toward the surface and away from the ar site. The D enantiomorph may keep the ester and aryl groups properly oriented with respect to the corresponding enzyme sites and is reactive; the L enantiomorph is less well suited for this and is far less reactive.

Experimental Section

L-(−)-Diethyl Malate (I). A solution of 10.0 g (0.075 mole) of L-malic acid (Nutritional Biochemicals Corp.), 40 g of absolute ethanol, and 4 g of concentrated hydrochloric acid was boiled on the steam bath for 4 hr and concentrated. The residue was distilled, leading to L-(−)-diethyl malate: 10.3 g (0.054 mole), 72% yield; bp 106–108° (2.5 mm); $n^{23}D$ 1.4340 (lit.[37] bp 91–92° (1 mm); $n^{25}D$ 1.4340); α_{obsd} −0.83°, 1 dm, 5.3% in acetone; $[\alpha]^{22}D$ −15.6° (lit.[38] −15.9° in acetone).

Anal. Calcd for $C_8H_{14}O_5$: C, 50.52; H, 7.42. Found: C, 50.66; H, 7.39 (Schwarzkopf).

L-(−)-Diethyl α-Acetoxysuccinate (II). L-(−)-Diethyl malate (5.0 g, 0.026 mole) was added at 0° with stirring to a solution of 6.0 g (0.059 mole) of freshly distilled acetic anhydride in 8 g (0.10 mole) of dry pyridine, stirred at 20° for 0.5 hr, and poured into 5% sulfuric acid. The solution was extracted with ether and the extract was dried, concentrated, and distilled, leading to 4.4 g (0.019 mole), 73% yield of L-(−)-diethyl α-acetoxysuccinate: bp 71–72° (0.15 mm); $n^{23}D$ 1.4260; α_{obsd} −25.50°, 1 dm, neat; d^{20}_4 1.030; $[\alpha]^{19}D$ −24.8° (lit.[39] $[\alpha]_{5780}$ −22.97°, neat); α_{obsd} −1.59°, 1 dm, 6.67% in absolute ethanol; $[\alpha]^{17}D$ −23.6°.

Anal. Calcd for $C_{10}H_{16}O_6$: C, 51.72; H, 6.79. Found: C, 52.00; H, 6.82 (Schwarzkopf).

D-(+)-Diethyl malate was prepared from D-malic acid (Nutritional Biochemicals Corp.) as described above for the L-enantiomorph: bp 65° (<0.1 mm); $n^{25}D$ 1.4333; α_{obsd} +0.76°, 1 dm, 5.4% in acetone; $[\alpha]^{22}D$ +13.95° (lit.[38] bp 125–127° (10 mm); $[\alpha]^{22}D$ +16.5° in acetone).

Anal. Found: C, 50.32; H, 7.35 (Schwarzkopf).

D-(+)-Diethyl α-acetoxysuccinate was prepared from 1.5 g (0.0079 mole) of D-(+)-diethyl malate as described for the L enantiomorph; 1.1 g (0.0058), 63% yield; bp 74–76° (0.15 mm); $n^{20.5}D$ 1.4274; α_{obsd} +1.76°, 1 dm, 7.70% in absolute ethanol; $[\alpha]^{19}D$ +22.9°.

Anal. Found: C, 51.81; H, 7.07 (Schwarzkopf).

DL-Diethyl malate was prepared from DL-malic acid as described above for the L enantiomorph: bp 100° (1.5 mm); $n^{21}D$ 1.4348 (lit.[40] bp 122–124° (12 mm)).

Anal. Found: C, 50.46; H, 7.44 (Schwarzkopf).

Infrared spectra of the DL-, D-(+)-, and L-(−)-diethyl malates in chloroform were identical, showing absorption bands at 2.85 μ (w), 3.4 (w), 4.1–4.3 (w), 5.75 (s), 6.25 (w), 6.8 (w), 7.3 (m), 7.7 (m), 8.15–8.5 (s), 9.1 (m), 9.8 (m), 10.8 (w), and 11.6–11.8 μ (w).

DL-Diethyl α-acetoxysuccinate was prepared from 5.0 g (0.026 mole) of DL-diethyl malate as described for the L compound: 3.3 g (0.14 mole), 54% yield; bp 129–130° (4 mm); $n^{21}D$ 1.4275.

Anal. Found: C, 51.98; H, 7.08 (Schwarzkopf).

Infrared spectra of the DL-, D-(+)-, and L-(−)-diethyl α-acetoxysuccinates were identical, showing absorption bands at 2.9 (w), 3.4 (w), 5.75 (s), 7.73 (m), 8.40 (s), 9.4–9.8 (m), 10.5–10.9 (w), and 11.7 μ (w).

DL-α-Ethyl Hydrogen α-Acetoxysuccinate. DL-Malic acid (1.0 g, 7.5 mmoles) and 2.7 ml of acetyl chloride were stirred at 40° for

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3 hr. The liquid was evaporated under vacuum, and the residue was triturated with dry ether, leading to 1.1 g (7.1 mmoles), 95% yield, of **DL-α-acetoxysuccinic anhydride**, mp 86–87°.

Anal. Calcd for $C_6H_6O_5$: C, 45.58; H, 3.83. Found: C, 45.27; H, 3.79 (Nagy).

This anhydride (0.30 g, 1.9 mmoles) was stirred with 0.5 ml of dry ethanol at 50° for 0.5 hr. Evaporation of the ethanol led to 0.35 g (1.7 mmoles), 89% yield, of α-ethyl hydrogen α-acetoxysuccinate, mp 70–71° (lit.[25] mp 70.4–71.2°).

The infrared spectrum in KBr showed the following bands: 3.35 (m), 5.7 (s), 5.85 (s), 6.75 (w), 6.9 (m), 7.15 (m), 7.3 (s), 7.4 (m), 7.7 (s), 8.05 (s), 8.2 (s), 8.4 (s), 8.9 (w), 9.1 (m), 9.35 (s), 9.75 (m), 9.9 (w), 10.3 (w), 10.5 (m), 10.75 (m), 11.45 (w), 11.7 (w), 12.7 (w), and 13.5 μ (w).

Hydrolysis of DL-Diethyl α-Acetoxysuccinate by α-Chymotrypsin. (i) DL-Diethyl α-acetoxysuccinate (0.658 g, 2.84 mmoles) and 0.074 g of α-chymotrypsin in 20 ml of 0.1 *M* NaCl were allowed to react under nitrogen in a pH-Stat at pH 7.2. After 2 hr, the reaction was complete, 2.84 ml of 1.0 *N* NaOH being consumed. The solution was dialyzed against 4 volumes of water and the dialysate lyophilized, leading to 0.570 g of residue. This residue was dissolved in water and stirred for 0.5 hr with 2.5 g of Dowex 50 (W-X8, sulfonic acid, 20–50 mesh, 5.2 mequiv/g). The solution was filtered off, the resin was washed with water, and the solutions were lyophilized, leading to 0.39 g (1.9 mmoles), equivalent to 84% yield of **DL-β-ethyl hydrogen α-acetoxysuccinate** (III), mp 71–72° from chloroform–hexane, α_{obsd} 0°, 4.25% in ethanol, mixture melting point with synthesized α-ethyl hydrogen α-acetoxysuccinate 52–65°. The infrared spectrum in KBr pellet showed peaks at 3.35 (m), 5.75 (s), 5.85 (s), 6.8 (w), 6.95 (m), 7.15 (m), 7.3 (m), 7.4 (w), 7.5 (w), 7.7 (w), 7.95 (m), 8.15–8.25 (s), 9.2 (m), 9.4 (m), 9.75 (m), 10.5 (w), 10.7 (w), 11.1 (w), 11.55 (w), 12.7 (w), and 14.45 μ (w).

Anal. Calcd for $C_8H_{12}O_6$: C, 47.06; H, 5.95. Found: C, 46.92; H, 5.90 (Nagy).

(ii) DL-Diethyl α-acetoxysuccinate (0.247 g, 1.16 mmoles) and 0.054 g of α-chymotrypsin in 20 ml of 0.1 *M* NaCl were allowed to react under nitrogen in a pH-Stat at pH 7.2. Hydrolysis of one ester group was 91% complete after 1.5 hr, 1.05 ml of 1.0 *N* NaOH being consumed. The solution was dialyzed against 4 volumes of water and the dialysate lyophilized, leading to 0.28 g of solid. This was dissolved in 15 ml of ethanol and 5 ml of water, and 0.22 g (0.83 mmole) of *p*-phenylphenacyl bromide was added. The solution was boiled for 3 hr, concentrated, and extracted with chloroform, leading to 0.24 g (0.59 mmole), equivalent to 63% yield of **DL-α-(*p*-phenylphenacyl) β-ethyl α-acetoxysuccinate** (IV), mp 88–89° from chloroform–isopropyl ether, α_{obsd} 0°, 5.4% in chloroform.

Anal. Calcd for $C_{22}H_{22}O_7$: C, 66.32; H, 5.57. Found: C, 65.99; H, 5.58 (Nagy).

Hydrolysis of L-(−)-Diethyl α-Acetoxysuccinate by α-Chymotrypsin. L-(−)-Diethyl α-acetoxysuccinate (0.62 g, 2.7 mmoles) and 0.066 g of α-chymotrypsin in 20 ml of 0.1 *M* NaCl were allowed to react in a pH-Stat at pH 7.2, 1.0 *N* NaOH being added from an automatic buret. Hydrolysis of one ester group was complete after 80 min. The solution was dialyzed, and the dialysate was lyophilized, leading to 0.567 g of residue. This residue was treated with 0.58 g (2.1 mmoles) of *p*-phenylphenacyl bromide as described above for the DL compound, leading to 0.77 g (1.93 mmoles), 72% yield of **L-(−)-α-(phenylphenacyl) β-ethyl α-acetoxysuccinate:** mp 87.5–88° from chloroform–isopropyl ether; α_{obsd} −0.71°, 5.1% in chloroform; $[\alpha]^{18}D$ −13.9.

Anal. Found: C, 66.01; H, 5.35 (Schwarzkopf).

Hydrolysis of D-(+)-Diethyl β-Acetoxysuccinate by α-Chymotrypsin. D-(+)-Diethyl α-acetoxysuccinate (0.58 g, 2.50 mmoles) and 0.083 g of α-chymotrypsin in 20 ml of water were allowed to react in a pH-Stat at pH 7.2, 1 equiv of 1.0 *N* NaOH being consumed in 1.5 hr. The solution was dialyzed and lyophilized, leading to 0.54 g of residue. This residue was dissolved in 5 ml of water, treated with 2 g of Dowex 50 for 0.5 hr, separated, and concentrated, leading to 0.44 g (2.2 mmoles), 88% yield of **D-(+)-β-ethyl hydrogen α-acetoxysuccinate**. The crystals were washed with cold isopropyl ether and petroleum ether: mp 52–54°; α_{obsd} +0.42°, 2.5% in ethanol; $[\alpha]^{18}D$ +16.8°.

Anal. Found: C, 47.09; H, 6.04 (Nagy).

Hydrolysis of DL-Diethyl Malate by α-Chymotrypsin. A solution of 0.91 g (4.8 mmoles) of DL-diethyl malate and 0.085 g of α-chymotrypsin in 15 ml of water was allowed to react in the pH-Stat at pH 7.2. Hydrolysis of 86% of one ester group occurred in 80 min, 4.13 ml of 1.0 *N* NaOH being consumed. The solution was dialyzed and lyophilized, leading to 0.72 g of the salt (3.9 mmoles), 81% yield. A portion of this (0.37 g, 2.0 mmoles) was dissolved in 25 ml of 4:1 ethanol–water, 0.55 g (2.0 mmoles) of *p*-phenylphenacyl bromide was added, and the solution was boiled for 3 hr. This solution was worked up as described above, leading to the derivative, 0.64 g (1.8 mmoles), 90% yield, mp 86–92°. After several crystallizations from chloroform–isopropyl ether, **DL-α-(*p*-phenylphenacyl) β-ethyl malate** was obtained with constant melting point at 101–102°.

Anal. Calcd for $C_{20}H_{20}O_6$: C, 67.40; H, 5.66. Found: C, 67.26; H, 5.81 (Nagy).

Hydrolysis of DL-Diethyl Malate by Alkali. DL-Diethyl malate (0.767 g, 4.04 mmoles) was dissolved in 15 ml of water and stirred in the pH-Stat, drawing 1 *N* NaOH and maintained initially at pH 7.2. Over a period of 2 days, 3.94 ml of the base was slowly consumed, corresponding to 97% of one ester group, the pH being raised to 8.6. Lyophilization led to 0.70 g of a salt which was dissolved in 30 ml of 2:1 ethanol–water and treated with 1.1 g (4.0 mmoles) of *p*-phenylphenacyl bromide under reflux for 3 hr. Work-up in the usual way led to 1.38 g of a mixture of products. Repeated crystallization from isopropyl ether led to **DL-di-*p*-phenylphenacyl malate:** 0.10 g (0.19 mmole), 5% yield; mp 196–197°.

Anal. Calcd for $C_{32}H_{26}O_7$: C, 73.50; H, 4.98. Found: C, 72.67; H, 5.03 (Nagy).

The mother liquors led to **DL-α-(*p*-phenylphenacyl) β-ethyl malate:** 0.30 g (0.85 mmole), 21% yield; mp 97–99°, not depressed on mixture with the sample obtained from the product of the enzymic hydrolysis. Infrared spectra of the two compounds were identical.

A suspension of 0.070 g (0.20 mmole) of this compound in 0.5 ml of acetic anhydride and 0.15 ml of acetyl chloride was stirred overnight and concentrated, leading to **DL-α-(*p*-phenylphenacyl) β-ethyl α-acetoxysuccinate:** 0.072 g (0.18 mmole), 90% yield; mp 88.5–89° from methylene dichloride–isopropyl ether, melting point undepressed on mixture with the sample prepared *via* enzymic hydrolysis of DL-diethyl α-acetoxysuccinate. Infrared spectra of the two samples were identical.

Hydrolysis of L-(−)-Diethyl Malate by α-Chymotrypsin. L-(−)-Diethyl malate (0.637 g, 3.35 mmoles) and 0.052 g of α-chymotrypsin in 15 ml of water were allowed to react in a pH-Stat at pH 7.2. Hydrolysis of one ester group was 94% complete after 1 hr, 3.14 ml of 1.0 *N* NaOH being consumed. The solution was dialyzed and lyophilized, and the residue was treated with *p*-phenylphenacyl bromide as described for DL compound, leading to **L-(+)-α-(*p*-phenylphenacyl) β-ethyl malate:** 0.30 g (0.84 mmole), 22% yield; mp 123–124° from methylene chloride–isopropyl ether; α_{obsd} +0.39°, 5.0% in chloroform; $[\alpha]^{18}D$ +7.8°.

Anal. Found: C, 67.33; H, 5.75 (Nagy).

A suspension of 0.060 g (0.17 mmole) of this compound in 0.5 ml of acetic anhydride and 0.15 ml of acetyl chloride was stirred until a clear solution resulted. It was concentrated in vacuum, leading to **L-(−)-α-(*p*-phenylphenacyl) β-ethyl α-acetoxysuccinate:** 0.059 g (0.15 mmole), 87% yield; mp 87–88° from methylene chloride–isopropyl ether, mp 87–88° on mixture with the sample prepared *via* enzymic hydrolysis of L-(−)-diethyl α-acetoxysuccinate; α_{obsd} −0.25°, 2.3% in chloroform; $[\alpha]^{18}D$ −10.9°.

Hydrolysis of D-(+)-Diethyl Malate by α-Chymotrypsin. A solution of 0.716 g (3.76 mmoles) of D-(+)-diethyl malate and 0.060 g of α-chymotrypsin in 15 ml of water was allowed to react in the pH-Stat at pH 7.2, 3.55 ml of 1.0 *N* NaOH being consumed in 2 hr, corresponding to 95% hydrolysis of one ester group. The solution was dialyzed and lyophilized, leading to 0.52 g (2.82 mmoles), 80% yield of residue. This was treated with 0.775 g (2.82 mmoles) of *p*-phenylphenacyl bromide, leading to 0.85 g (2.38 mmoles), 83% yield, of crude derivative, mp *ca.* 112°, 123–124° after several recrystallizations from methylene chloride–isopropyl ether; α_{obsd} −0.34°, 3.7% in chloroform; $[\alpha]^{18}D$ −9.2°, **D-(−)-α-(*p*-phenylphenacyl) β-ethyl malate.**

Anal. Found: C, 67.22; H, 5.91 (Nagy).

A portion of this derivative, 0.090 g (0.25 mmole), was treated with acetic anhydride and acetyl chloride as described above for the L compound, leading to **D-(+)-α-(*p*-phenylphenacyl) β-ethyl α-acetoxysuccinate:** 0.094 g (0.24 mmole), 95% yield; mp 86–88° from methylene chloride–isopropyl ether; α_{obsd} +0.24°, 4.5% in chloroform; $[\alpha]^{22}D$ +5.35°. The infrared spectrum was identical with that of the L-(−) compound above.

Kinetics of Hydrolysis of D-(+)-, L-(−)-, and DL-α-Acetoxysuccinate by α-Chymotrypsin. The weighed quantity of ester was added to 19 ml of 0.1 *M* NaCl under nitrogen at 25°, and the solution was brought to pH 7.2 in a pH-Stat. Consumption of 0.10 *N* NaOH due to hydrolysis by water was followed for about 10 min and varied between 1 and 6% of the enzymic rate. The enzyme

(Worthington Biochemicals Corp., Lot No. 6082-3, 12% water, 2 mg in 1 ml of water, 0.35×10^{-5} *M*) was added. The pH was restored to 7.2, the consumption of 0.10 *N* NaOH was followed, and the corrected initial zero-order enzymic rates were determined. Data are summarized in Table I.

Kinetics of hydrolysis of D-(+)-, L-(−)-, and DL-diethyl malate by α-chymotrypsin (0.15×10^{-5} *M*) were followed as described above for the acetoxysuccinates. Corrections for the nonenzymic hydrolysis, 2–6%, were applied, and corrected enzymic rates at the several concentrations are summarized in Table II.

Stereospecificity in Hydrolysis by α-Chymotrypsin of Esters of α,α-Disubstituted Acetic and β,β-Disubstituted Propionic Acids[1]

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Abstract: Ethyl α-phenyl-α-acetamidoacetate is hydrolyzed by α-chymotrypsin with L specificity, $k_{cat} = 0.89\ sec^{-1}$, $K_{m,app} = 0.0033\ M$. Ethyl α-phenyl-α-benzamidoacetate is also hydrolyzed with L specificity. Ethyl mandelate is hydrolyzed without stereospecificity. The D specificity in hydrolyses of ethyl β-phenyl-β-acetamidopropionate and of ethyl β-phenyl-β-hydroxypropionate is accounted for in terms of the reactive conformation of the substrates and the correspondence of D-β and L-α substituents.

In the two preceding papers[2,3] the conformations of reacting substrates and the modes of their association with α-chymotrypsin have been described. α-Substituted, β-substituted, and α,β-disubstituted derivatives of ethyl propionate have been considered as model small molecule substrates. In hydrolyses proceeding with normal L stereospecificity, the α substituent associates at the α-acylamido site, am, the β substituent at the β-aryl site, ar, and the α hydrogen at a site of restricted volume, h. The D enantiomorphs may react under the following circumstances: (1) when there is no β substituent and the α substituent prefers to associate at the ar site, as in ethyl α-benzoyloxypropionate,[4] $CH_3CH(OCOC_6H_5)CO_2C_2H_5$; (2) when the α and β substituents may each associate at either the am or ar sites, as in diethyl α-acetoxysuccinate[3] and in effect in diethyl β-acetoxyglutarate;[5] (3) when the α substituent may associate at either the am or h site, as in ethyl β-phenyl-α-hydroxypropionate,[6,7] ethyl β-phenyl-α-chloropropionate,[6,7] and diethyl α-hydroxysuccinate.[3] This view of substrate–enzyme associations, combined with the transoid conformation of β substituent and hydrolyzing ester or amide group, and the location of the β substituent, directed into the enzyme, seems to be generally useful for α,β-disubstituted propionates and to have predictive value. It may also account for the D specificity of the cyclic substrate 1-keto-3-carbomethoxytetrahydroisoquinoline[8] which in effect falls into classes 1 and 3, above.

We have previously reported[9] that two β,β-disubstituted esters, ethyl β-phenyl-β-hydroxypropionate and ethyl β-phenyl-β-acetamidopropionate, are hydrolyzed slowly by α-chymotrypsin with remarkable D specificity. As to the corresponding α,β-disubstituted compounds, ethyl α-acetamido-β-phenylpropionate, the β-phenylalanine derivative,[10] shows essentially complete L specificity, and ethyl α-hydroxy-β-phenylpropionate also shows more rapid hydrolysis of the L enantiomorph. It was not clear whether the D specificity in the hydrolysis of the β,β-disubstituted compounds arose because the hydroxyl and acetamido substituents were β to the hydrolyzing ester group or because they were α to the important aryl substituent. In the β-substituted diethyl glutarates, the β-hydroxy[11] and β-acetamido[12] derivatives both hydrolyze with L specificity. In these compounds the hydroxyl and acetamido groups, while β to the hydrolyzing ester group, are also β to the second carbethoxyl, which associates at the ar site. In the latter sense the hydroxyl and acetamido groups could and did occupy the α am site and led to normal L specificity.

It seemed of interest to reexamine, with the procedures elaborated in the previous article, the D specificity of hydrolysis by α-chymotrypsin of β,β-disubstituted propionates, and to study the nature of the stereospecificity of hydrolysis of corresponding α,α-disubstituted acetates.

Results

Ethyl DL-α-phenyl-α-acetamidoacetate and the D-(−) enantiomorph were prepared from the commercially available DL- and D-(−)-phenylglycines by

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