binding capacity of 0.10%, and absorptivity of the Fe-transferrin at 465 mμ of 0.42, are lower than the figures of 0.125% and 0.57, respectively, reported for crystalline human transferrin by Surgenor *et al.*, (1949) and the 0.126% and 0.48 to 0.55 found for crystalline swine transferrin by Laurell (1953). Laurell has also mentioned the difficulty in preparing swine transferrin free from a heme-containing globulin characterized by high absorptivity at 408 mμ. A small amount of a similar impurity is present in T_{2a}, and our values for iron-binding capacity and absorptivity at 465 mμ may be a little low for this reason. It is also possible, of course, that these constants are characteristically lower in the bovine variety.

The problem of preparing a purified bovine blood β-globulin (transferrin) which can be considered homogeneous by current standards is further complicated by the occurrence of genetic variants of this protein (Ashton, 1958, 1959; Gahne, 1961). We have not attempted to deal with this aspect of the problem in this paper, since we have used mixed herd milk and, we assume, pooled blood as sources of protein. As has been stated previously, our conclusion regarding the nonidentity of "red protein" and transferrin is in accord with the results reported by Derechin and Johnson (1962).

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Action of α-Chymotrypsin on Diethyl *N*-Acetylaspartate and on Diethyl *N*-Methyl-*N*-Acetylaspartate*

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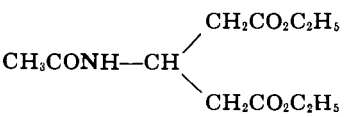
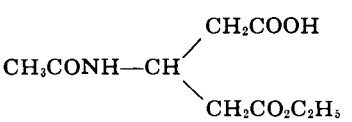
Study of glutarate esters had indicated that a β-carbalkoxyl group would be an activating substituent in hydrolyses catalyzed by α-chymotrypsin. L(−)Diethyl-*N*-acetylaspartate is hydrolyzed effectively by α-chymotrypsin, K_m = 0.023 M, k_3 = 22 sec^{-1}, leading to L(+)β-ethyl-α-hydrogen-*N*-acetylaspartate. The L-enantiomorph is also hydrolyzed rapidly from the DL material, leading to L(+)β-ethyl-α-hydrogen-*N*-acetylaspartate and D(+)diethyl-*N*-acetylaspartate. The D-enantiomorph does not inhibit hydrolysis of the L, and is itself hydrolyzed very slowly. Diethyl-*N*-methyl-*N*-acetylaspartate is not hydrolyzed by α-chymotrypsin. The results are compared with those for derivatives of alanine and β-phenylalanine, placing L(−)diethyl-*N*-acetylaspartate among the good substrates for α-chymotrypsin. It is proposed that the β-carbethoxyl group of the aspartate associates at the β-aryl site of α-chymotrypsin.

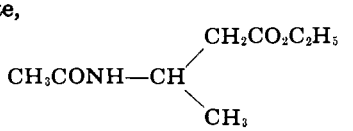
We have reported (Cohen and Khedouri, 1961a) that diethyl-β-acetamidoglutarate,



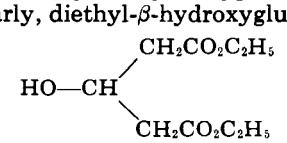
is hydrolyzed by α-chymotrypsin, slowly but with stereospecificity, leading to (+)ethyl-hydrogen-β-acetamidoglutarate,



of high optical purity, and this hydrolysis probably proceeds in the L sense (Cohen and Khedouri, 1961b). The related asymmetric compound, ethyl-*dl*-β-acetamidobutyrate,



is not hydrolyzed by α-chymotrypsin (Cohen *et al.*, 1961). Similarly, diethyl-β-hydroxyglutarate



is slowly hydrolyzed by α-chymotrypsin, in the L sense and with high stereospecificity, while the related asymmetric compound, ethyl-*dl*-β-hydroxybutyrate,

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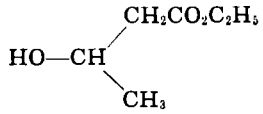
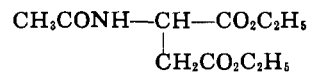
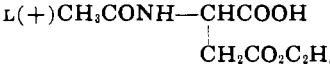


is hydrolyzed more slowly and without stereospecificity (Cohen and Khedouri, 1961b). In these cases the second carbethoxyl group of the glutarates, located β to the developing center of asymmetry, led to increased rates of hydrolysis and stereospecificity. The second carbethoxyl group may lead to improved association with the enzyme, perhaps much like that due to the β-aryl groups of related derivatives of tyrosine and β-phenylalanine (Neurath and Schwert, 1950). It seemed of interest to examine the effect of the β-carbethoxyl in a potentially more reactive situation, in a substrate in which the center of asymmetry and acetamido group are α to the hydrolyzing ester group, i.e., to examine the kinetics and stereospecificity of hydrolysis of diethyl-N-acetylaspartate, DEAA,[1]



and to compare the results with those for ethyl-N-acetylalaninate and ethyl-N-acetyl-β-phenylalaninate. We now report that L-diethyl-N-acetylaspartate is hydrolyzed quite effectively by α-chymotrypsin.

Diethyl - N - Acetylaspartate, DEAA.—L(−)DEAA $[\alpha]_D^{20}$ − 10.6°, 5% in absolute ethanol, and DL-DEAA were prepared by esterification of L and DL aspartic acids, followed by acetylation of the esters. Preliminary experiments indicated that L(−)DEAA was hydrolyzed very rapidly by α-chymotrypsin. Treatment of 1.2 g of L(−)DEAA with 10 mg of the enzyme led to complete hydrolysis of one ester group within a few minutes and isolation of the product of hydrolysis of the α-carbethoxyl group, L(+)β-ethyl-α-hydrogen-N-acetylaspartate, β-EHAA,

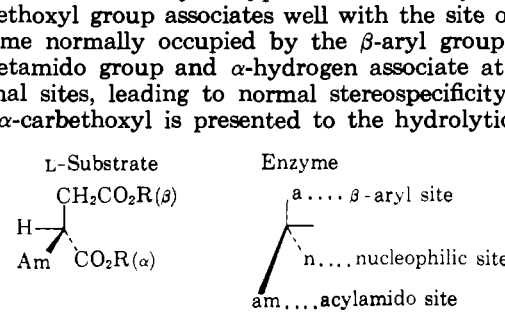


$[\alpha]_D^{20.8}$ + 14.5°, 5%in absolute ethanol. The latter had been synthesized previously from L-aspartic acid and its structure demonstrated (Cohen and Khedouri, 1961b). It was at this time further purified. The product of the enzymatic hydrolysis was identical with the synthesized material.

DL-DEAA was also hydrolyzed rapidly by α-chymotrypsin, the reaction essentially stopping after 0.5 equivalent of alkali was consumed. Unhydrolyzed D(+)DEAA was recovered in good (68%) yield, with high optical purity, $[\alpha]_D^{20.8}$ + 10.9°, 7% in absolute ethanol. The hydrolysis product, isolated in 74% yield, was again, L(+)β-EHAA, $[\alpha]_D^{20.8}$ + 14.6°, 5% in absolute ethanol, + 7.9°, 6% in acetone. This enzymatic reaction thus provides a convenient access to L and D derivatives of N-acetylaspartic acid.

The kinetics of hydrolysis of L(−) and DL-DEAA at *p*H 7.2, catalyzed by 0.02 mg/ml of α-chymotrypsin in 0.1 M NaCl, were studied. Pseudo zero-order rates are listed in Table I. Linear double reciprocal plots were obtained (Lineweaver and Burk, 1934), $1/V$ vs. 1/S, and from them the kinetic parameters K_m and k_3 were calculated. The results are given in Table II along with data for L-ethyl-N-acetylalaninate and L-ethyl-N-acetyl-β-phenylalaninate.

While the β-carbethoxyl group has not led to as favorable values of K_m and k_3 as those of the derivative of phenylalanine, the kinetic parameters are favorable indeed; L-DEAA is far more reactive than L-ethyl-N-acetylalaninate and may be placed among the good substrates for α-chymotrypsin. Presumably the β-carbethoxyl group associates well with the site on the enzyme normally occupied by the β-aryl group; the α-acetamido group and α-hydrogen associate at their normal sites, leading to normal stereospecificity, and the α-carbethoxyl is presented to the hydrolytic site.



The nature of the β-carbethoxyl group allows it to associate at the β-aryl site and its dimensions are such as to place the α-carbethoxyl reasonably convenient to the nucleophilic site, leading to high reactivity. The carbethoxy group is of interest as an activating group which may impart more water solubility than the aryl groups, and allow study of a number of interesting substrates. The α-acetoxyl group, in ethyl-α-acetoxypropionate, may also associate at the β-aryl site, but since it occupies the position in the substrate of the usual acylamido group, inversion of stereospecificity may result (Cohen *et al.*, 1962).

An interesting aspect of the kinetics, revealed in Table II, is that k_3 is essentially the same for L- and

TABLE I

RATES OF HYDROLYSIS OF L- AND DL-DIETHYL-N-ACETYLASPARTATE (DEAA) BY α-CHYMOTRYPSIN (0.02 MG/ML), 0.1 M NaCl, *p*H 7.2, 25°

Compound	10^3 S (mole/liter)	10^6v (mole/liter/sec)
L-DEAA	1.45	1.05
	1.73	1.20
	2.12	1.64
	2.62	1.83
	2.97	1.85
	4.13	2.84
	5.19	3.18
	10.19	5.35
DL-DEAA	1.47	0.54
	1.64	0.58
	1.89	0.70
	2.36	0.90
	2.97	1.08
	3.65	1.28
	5.10	1.68
	7.88	2.53

TABLE II

KINETIC CONSTANTS FOR SOME HYDROLYSES CATALYZED BY α-CHYMOTRYPSIN

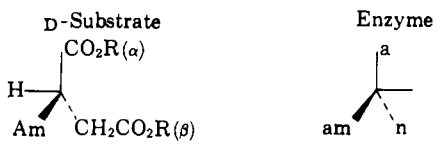
Substrate	K_m (mole/liter)	k_3 (sec^{-1})
L-Diethyl-N-acetylaspartate[a]	0.023	22
DL-Diethyl-N-acetylaspartate[a]	0.044	21
L-Ethyl-N-acetylalaninate[b]	0.25	0.34
L-Ethyl-N-acetylphenylalaninate[c]	0.0019	120

[a] 0.1 M NaCl, 25°, *p*H 7.2, this work. Errors in K_m and k_3 are estimated to be ±0.002 and ±2, respectively. [b] S. Kreisler and S. G. Cohen, unpublished results, 0.1 M NaCl, 25°, *p*H 7.8. [c] Taken from Hammond and Gutfreund (1955), 0.1 M NaCl, 25°, *p*H 7.2.

[1] Abbreviations used in this work: DEAA, diethyl-N-acetylaspartate; β-EHAA, β-ethyl-α-hydrogen-N-acetylaspartate.

DL-DEAA, while K_m for the DL material is approximately twice that of the L. Examination of the simple plots of the data for these two materials of Table I indicates that if the rates of hydrolysis of the DL material are plotted against one-half its concentration, i.e., against the concentration of the L compound present, the points fall essentially directly on the plot for the data for the pure L compound. This is equivalent to the relation of the values of the kinetic parameters referred to above. This failure of the D compound to act as an inhibitor may indicate that, although the β-carbethoxyl group may associate at the β-aryl site, firm association of this substrate with the enzyme may require contributions from several of the groups, which are not readily available from the D compound. It may be readily displaced by the L, and appears neither to inhibit nor to hydrolyze appreciably in the presence of the L. On the other hand, compounds with a β-aryl group may show strong association almost through that group alone, and inhibition by D-enantiomorphs containing β-aryl groups and by relatively simple aromatic compounds is observed (Foster and Niemann, 1955).

When the D-enantiomorph of DEAA, 2.4×10^{-3} M, was subjected alone to α-chymotrypsin, under the same conditions as the L, slow hydrolysis occurred, about 4% as great as that of the L, after correction for the water hydrolysis. This reaction was not studied further. High optical purity of the recovered unhydrolyzed ester and the half acid in the hydrolysis of DL-DEAA had indicated high stereospecificity. It is possible that the slow hydrolysis of the D-enantiomorph when studied alone involves in part the β- rather than the α-carbethoxyl group. Association of this enantiomorph with the enzyme, with the α-hydrogen and acetamido groups at their normal sites, would place the β-carbethoxyl in the region of nucleophilic site, but not in the exact position that may be occupied by the α-carbethoxyl of the L-enantiomorph.



Hydrolysis of diethyl-β-acetamido-glutarate (Cohen and Khedouri, 1961a) indicates that a β-carbethoxyl may be hydrolyzed slowly by α-chymotrypsin, but in that case there was present a second, activating, β-carbethoxyl group.

DL-*Diethyl-N-Methyl-N-Acetylaspartate.*—Substitution of an oxygen atom in ethyl-α-acetoxypropionate for an NH group in ethylacetylalaninate led to decrease in reactivity and inversion of stereospecificity (Cohen *et al.*, 1962), indicating that a hydrogen bonding association of the acylamido group with the enzyme may be important. To examine this, the *N*-methyl derivative of diethyl-*N*-acetylaspartate was prepared by addition of methylamine to diethyl maleate, followed by acetylation. Treatment of 0.052 g of this material with 0.020 g of α-chymotrypsin led to no consumption of alkali beyond the small amount due to hydrolysis of the material by water and to hydrolysis of the enzyme. *N*-Methylation apparently prevents *effective* association of this substrate with the enzyme. *N*-Methylation of methyl-*N*-acetyltyrosinate has also been reported to lead to a very unreactive substrate (Hein and Niemann, 1961).

Experimental

α-Chymotrypsin was obtained from Worthington Biochemical Corporation, three times recrystallized. Samples were dried to constant weight at 120° for assay of protein content. The molecular weight was assumed to be 25,000 for calculation of kinetic parameters.

Optical rotations were determined in a Zeiss-Winkel polarimeter and read to ±0.02°.

L-*Diethyl-N-Acetylaspartate.*—L-aspartic acid was obtained from Nutritional Biochemicals Co., $[\alpha]_D^{21}$ + 19.6°, 6.9% in N HCl. L-Aspartic acid, 25 g (0.19 mole), was suspended in 500 ml of absolute ethanol, and dry hydrogen chloride was passed in until a solution resulted. This was boiled under reflux for 2 hours, and taken to dryness in vacuum, leaving a residue mp 93–95°. This was heated on the steam bath with 50 ml of acetic anhydride and 50 g of sodium acetate for 1.5 hours, cooled, and treated with 100 ml of chloroform. Precipitated salt was removed, and the filtrate was concentrated in vacuum and distilled, leading to L(−)diethyl-*N*-acetylaspartate, bp 132–133° (0.2 mm), n_D^{25} 1.4516, $[\alpha]_D^{29}$ −10.64°, 5.62% in ethanol, reported $[\alpha]_D^{19}$ −9.08 (Freudenberg and Noe, 1925.) The infrared spectrum in chloroform showed peaks at 3.05 μ (m),[2] 3.35(m), 5.75(s), 6.02(s), 6.5(s), 7.3(s), 7.75 (s), 8.2–8.5(s), 9.15(m), 9.72(s), 10.5(w), 11.6(w).

Anal. Calcd. for $C_{10}H_{17}NO_5$: C, 51.94; H, 7.41; N, 6.06. Found: C, 51.90; H, 7.50; N, 5.95 (Dr. C. Fitz).

DL-*Diethyl-N-Acetylaspartate.*—DL-Aspartic acid (Mann Research Laboratories, 10 g, 0.075 mole) was suspended in 200 ml of absolute ethanol, and dry hydrogen chloride was passed until a solution resulted. This was boiled under reflux for 2 hours, and concentrated; the oily residue was dissolved in chloroform and washed with saturated aqueous potassium carbonate and with water. The chloroform solution was saturated with hydrogen chloride, concentrated to 10 ml, and diluted with ether, leading to DL-diethyl-aspartate hydrochloride, mp 99–100°, 7.04 g (0.031 mole), 42% yield. This was dissolved in 30 ml of water, neutralized with sodium acetate, and stirred at room temperature for 3 hours with 25 ml of acetic anhydride. The solution was concentrated and the residue was distilled, leading to DL-diethyl-*N*-acetyl-aspartate, bp 120° (0.1 mm), n_D^{25} 1.4524, 4.47 g. (0.019 mole), 62% yield.

Anal. Found: C, 52.19; H, 7.30; N, 5.96 (Dr. S. M. Nagy).

L(+)β-*Ethyl-α-Hydrogen-N-Acetylaspartate.*—This material, previously prepared (Cohen and Khedouri, 1961b), was recrystallized from acetone–petroleum ether, mp 120–121°, $[\alpha]_D^{20.8}$ + 7.78°, 1.03% in acetone.

Hydrolysis of L(−)*Diethyl-N-Acetylaspartate by* α-*Chymotrypsin.*—(1) To 20 ml of a solution in a *p*H stat containing 0.12 mg of the enzyme at *p*H 7.8 was added 48.2 mg of the substrate, 0.1 N alkali being added from an automatic burette. Fifty per cent hydrolysis of one ester group required 5 minutes, 100%, 25 minutes.

(2) To 20 ml of a solution containing 10 mg of the enzyme and 1.5 ml of 0.1 M Na_2HPO_4 was added 1.21 g (0.0052 mole) of the substrate. Reaction was too rapid for the automatic burette and was complete in a few minutes. The solution was brought to *p*H 2, taken to dryness in vacuum, and extracted with acetone, leading to L(+)β-ethyl-α-hydrogen-*N*-acetyl-aspartate, mp 120–121°, from acetone–petroleum ether, mixed with synthesized sample, mp 120–121°, $[\alpha]_D^{20.8}$ + 14.48°, 5.29% in absolute ethanol. The

[2] Letters in parentheses are: (m), medium; (s), strong; (w), weak.

infrared spectrum in chloroform showed peaks at 2.97 μ (m), 3.45(s), 5.75(m), 6.20(m), 6.52(m), 6.87(s), 7.3(s), 7.4(w), 7.7(w), 7.9(w), 8.2(s), 8.45(s), 8.8(w), 9.75(m), 10.15(w), 10.35(w), 10.5(w), 11.1(w), 11.3(w), 11.6(m), 12.5(w). It was identical with that of the synthesized sample.

Hydrolysis of DL-*Diethyl-N-Acetylaspartate by α-Chymotrypsin.*—The substrate, 1.30 g (0.0056 mole), was hydrolyzed in 20 ml of 0.1 M sodium chloride containing 0.050 g of α-chymotrypsin at *p*H 7.2 in a *p*H stat with automatic burette delivering 1 N sodium hydroxide. One-half an equivalent of alkali, 2.8 ml, was required in a few minutes and then the reaction essentially stopped. The solution was lyophilized and the residue was extracted with several 20-ml portions of acetone. The extracts were dried over magnesium sulfate, concentrated, and distilled, leading to D(+)*diethyl-N-acetylaspartate*, bp 114–115° (0.1 mm), n_D^{25} 1.4541, $[\alpha]_D^{20.8}$ + 10.89°, 6.98% in absolute ethanol, 0.44 g (0.0019 mole), 68% yield. The infrared spectrum was identical in all respects with that of the synthesized L-enantiomorph.

Anal. Found: C, 51.90; H, 7.50; N, 6.20 (Dr. C. Fitz).

The original residue, after extraction with acetone, was dissolved in a little water, brought to *p*H 2 with hydrochloric acid, concentrated, and extracted with acetone, leading to L(+)β-ethyl-α-hydrogen-*N*-acetylaspartate, mp 119–120° from acetone–petroleum ether, 0.42 g (0.0021 mole), 74% yield, $[\alpha]_D^{20.8}$ + 14.57°, 5.29% in absolute ethanol, $[\alpha]_D^{20.8}$ + 7.9°, 5.96% in acetone. The infrared spectrum was identical with that of the acid obtained by hydrolysis of the L substrate.

Kinetic Procedure.—Twenty ml of 0.1 M sodium chloride containing 0.4 mg of α-chymotrypsin was pipetted into the reaction flask and allowed to equilibrate under nitrogen with magnetic stirring at *p*H 7.2, 25 ± 0.1°. The substrate, weighed into a glass container, was dropped into the reaction vessel, a timer was started; *p*H was maintained by a Radiometer Titrator, Model TTT1b, which delivered 0.1 N sodium hydroxide from an automatic burette. Readings of alkali uptake vs. time were taken and plotted. Pseudo zero-order rates were calculated and are summarized in Table I. Nonenzymatic hydrolysis of the aspartate was about 2% of the enzymatic rate and corrected values are given in Table I. Consumption of alkali by the enzyme was negligible. Runs carried out at *p*H 7.5 and 7.8 gave the same rates as at 7.2.

DL-*Diethyl-N-Methyl-N-Acetylaspartate* (Lynch, 1948).—Dry methylamine was passed into 30 ml of *t*-butyl alcohol for 5 minutes and an aliquot was titrated against *N*-hydrochloric acid. One equivalent of diethyl maleate, 8.7 g (0.050 mole), was added to 20 ml of this solution with cooling, and the solution was allowed to stand overnight. The solution was concentrated and the residue was distilled, leading to diethyl-*N*-methylaspartate, bp 75° (0.1 mm), n_D^{26} 1.4301, 8.7 g (0.043 mole), 85% yield. A portion of this, 2.0 g (0.010 mole), was stirred with 10 ml of acetic anhydride, warmed briefly, and left at room temperature for 5 hours. The solution was concentrated and the residue was distilled in vacuum, bp 115° (0.1 mm), n_D^{23} 1.4505, 2.2 g (0.0091 mole), 91% yield.

Anal. Calcd. for $C_{11}H_{19}NO_5$: C, 53.86; H, 7.81; N, 5.71. Found: C, 54.08; H, 7.87; N, 6.25 (A. Bernhardt).

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