This is what would be expected on the basis of the Michaelis constants of the respective substrates.

In addition, the same compounds which are competitive inhibitors of the deamination reaction (Cory and Suhadolnik, 1965) are also inhibitors of the dechlorination reactions. Presumably this inhibition is also of the competitive type.[1] The dechlorination reaction was shown not to be reversible. This result is in agreement with the data reported by Kalckar (1947) on the irreversibility of the deamination reaction.

It is concluded that the same enzyme and the same active site on adenosine deaminase is involved in both the dechlorination and deamination reactions. That 6-chloropurine ribonucleoside can be converted to inosine and Cl^- by adenosine deaminase could complicate studies involving the use of this compound as an anticancer agent.

[1] The nature of the assay system for the dechlorination of 6-chloropurine ribonucleoside, that is, the change in absorbance at 250 and 265 mμ, the relatively high K_m for 6-chloropurine ribonucleoside, and the high extinction coefficients of both the substrates and inhibitors at these wavelengths, makes it technically difficult to determine the type of inhibition imposed on the dechlorination reaction.

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The Primary Specificity of α-Chymotrypsin. Interaction with Acylated Derivatives of D-Valine Methyl Ester and D-Norvaline Methyl Ester*

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ABSTRACT: The chymotrypsin-catalyzed hydrolysis of acetyl-L-leucine methyl ester is inhibited by derivatives of acylated D-valine and D-norvaline methyl esters. Competitive inhibition constants for the D-valine derivatives are acetyl $> 195 \times 10^{-3}$ M, chloroacetyl $= 169 \times 10^{-3}$ M, and benzoyl $= 5.2 \times 10^{-3}$ M. For the corresponding D-norvaline compounds the values are 111×10^{-3} M, 58.6×10^{-3} M, and 1.77×10^{-3} M, respectively. The chymotrypsin-catalyzed hydrolysis of *N*-benzoyl-L-norvaline methyl ester follows Michaelis-Menten kinetics with $K_0 = 0.85 \pm 0.16 \times 10^{-3}$ M and $k_0 = 2.45 \pm 0.14$ sec^{-1}. The values of the ratios of binding constants of the L and D enantiomers [K_0(L)/K_0(D)] support earlier conclusions that the acetyl- and chloroacetyl-L-valine and -L-norvaline methyl esters are bound predominantly in a productive mode, whereas the corresponding benzoyl derivatives of valine are mainly bound in a nonproductive manner. The data for the benzoyl norvaline methyl esters suggest that these binding constants are also controlled by more than one mode of combination. As observed previously in the L series, the isopropyl side chain of the D-valine esters offers greater steric hindrance to binding than does the *n*-propyl group of the D-norvaline compounds.

The primary specificity of chymotrypsin, that is, its catalytic behavior toward compounds containing only a single amino acid residue or related structures, has been the subject of intensive study during the past 15 years (Niemann, 1964). A general correlation between substrate and enzyme specificity has been developed (Hein and Niemann, 1961, 1962) and has since been applied successfully to explain the catalytic

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behavior of chymotrypsin toward a wide range of substrates (Jones *et al.*, 1965; Wolf and Niemann, 1963; Cohen *et al.*, 1962). The description of the specificity pattern relates the observed kinetic constants, K_0 and k_0, for the enzyme-catalyzed hydrolysis of model substrates to the structure of the compounds, for the many cases where the enzyme-catalyzed hydrolysis can be described by the familiar Michaelis-Menten equation (equation 1).

$$-d[S]/dt = d[P]/dt = k_0[E][S]/(K_0 + [S]) \quad (1)$$

A significant feature of the correlation has been the recognition of the existence of unproductive binding modes between substrate and enzyme (Hein and Niemann, 1961, 1962; Cohen and Weinstein, 1964). Part of the evidence for the involvement of such "wrong-way" binding modes comes from observed decreases in both kinetic parameters, K_0 and k_0, for a particular member of a series of structurally related compounds. For example, it has been argued (Hein and Niemann, 1961, 1962) that in a series of acylated valine methyl esters the *N*-benzoyl compound is bound to a significant extent in a "wrong-way" mode relative to the *N*-acetyl and *N*-chloroacetyl compounds because both kinetic constants for the *N*-benzoyl compound are substantially lower than the corresponding constants for the aliphatic analogs.

This type of argument in favor of the "wrong-way" binding concept is, of course, only suggestive, not conclusive. The concept is supported by the observation of experimental results predicted from it (Rapp and Niemann, 1963); its efficacy in the development of a quantitative correlation between structure and reactivity (C. Hamilton, C. Niemann, and G. S. Hammond, 1965, paper in preparation), and by a limited amount of spectroscopic evidence (Burr and Koshland, 1964).

A particular limitation of the type of argument mentioned here is that it assumes that K_0 values for a series of substrates reflect true binding constants between substrate and enzyme. It is not necessary that K_0 values actually approximate equilibrium binding constants, but, for the members of a series to be compared, it is essential that the observed variations reflect changes in that fraction of K_0 which measures enzyme-substrate binding.

The nonequilibrium character of K_0 for substrates of chymotrypsin has recently been stressed by Bender and his co-workers (e.g., Zerner and Bender, 1964). For esters of the best substrates, deacylation of an acyl enzyme intermediate may be rate limiting, so that $K_0 = (k_3/k_2)K_s$, where K_s is the true binding constant. This situation does not hold for esters of acetylvaline since k_0 varies with the nature of the ester moiety (Waite and Niemann, 1962). For these valine esters either k_2 and k_3 are of the same order of magnitude, in which case $K_0 = (k_3/(k_2 + k_3))K_s$, or $k_2 < k_3$, in which case $K_0 = K_s$.

The ambiguity in the interpretation of K_0 for valine derivatives and similar compounds can be at least partially resolved by studies on D isomers of these specific substrates. If the observed variations in K_0 values reflect differences in binding, similar trends should be noted in comparing a series of D-amino acid isomers. For these latter compounds the observed inhibition constants, K_I, can be interpreted operationally as equilibrium constants when no product formation is detected.

A further reason for studying the inhibition of α-chymotrypsin-catalyzed reactions by D-amino acid derivatives is that the tetradentate binding envisaged (Niemann, 1964) predicts the behavior of D isomers from the kinetic properties of corresponding L antipodes. The present investigation describes the interaction of three acylated D-valine methyl esters and two acylated D-norvaline methyl esters with α-chymotrypsin and compares the results with information obtained for the corresponding L isomers (Waite and Niemann, 1962; Jones and Niemann, 1962).

Experimental

Chloroacetyl-D-valine Methyl Ester. A solution of 20 g of D-valine methyl ester hydrochloride in 15 ml of water and 50 ml of ether was treated with a solution of 5.3 g of sodium hydroxide dissolved in 5 ml of water. The ethereal phase was separated and the aqueous layer was extracted with 2 × 50 ml of ether. The combined ether extracts were dried over magnesium sulfate and evaporated to a volume of 100 ml. The ether solution was cooled to 0° and 7.0 g of chloroacetyl chloride in 25 ml of anhydrous ether was added dropwise with stirring during 30 minutes, the apparatus being protected from atmospheric moisture. The reaction mixture was filtered to remove 9.7 g of precipitated D-valine methyl ester hydrochloride and the filtrate was evaporated *in vacuo*. The resulting 7.0 g of viscous oil was recrystallized from isopropyl ether–pentane, giving needles, to yield 5.9 g of chloroacetyl-D-valine methyl ester, mp 45–56°, $[\alpha]_D^{25°}$ 46.1 ± 2.0° (*c*, 2.6% in water).

Anal. Calcd for $C_8H_{14}ClNO_3$ (207.7): C, 46.3; H, 6.8; N, 6.8. Found: C, 46.3, 46.4; H, 6.7, 6.8; N, 6.9, 6.9.

Acetyl-L-leucine Methyl Ester. Acetyl-L-leucine methyl ester, prepared in 55% yield according to the method of Applewhite *et al.* (1958), was identical with the product obtained previously by Jones *et al.* (1962).

Acetyl-D-valine Methyl Ester. The method of Brenner and Huber (1953) was used to esterify 20 g of D-valine; acetylation of 7.5 g of the ester hydrochloride as described by Applewhite *et al.* (1958) gave 4.8 g of the product, mp 62°, $[\alpha]_D^{25°}$ 48.3 ± 0.5° (*c*, 5% in water).

Anal. Calcd for $C_8H_{15}NO_3$ (173.2): C, 55.5; H, 8.7; N, 8.1. Found: C, 55.6; H, 8.8; N, 8.1.

Benzoyl-D-valine Methyl Ester. D-Valine methyl ester hydrochloride was benzoylated in a chloroform-water system with benzoyl chloride as described by Applewhite *et al.* (1958). The product was recrystallized from isopropyl ether–hexane to give benzoyl-D-valine

methyl ester as silky needles, mp 111°, $[\alpha]_D^{25°}$ − 44.4 ± 1.5° (*c*, 1% in chloroform).

Anal. Calcd for $C_{13}H_{17}NO_3$ (235.3): C, 66.4; H, 7.3; N, 5.9. Found: C, 66.5; H, 7.3; N, 5.9.

*N-Acetyl-*D*-norvaline Methyl Ester.* D-Norvaline methyl ester hydrochloride was prepared by the method of Brenner and Huber (1953). Acetylation was performed as described for benzoyl-D-valine methyl ester. The product was recrystallized from isopropyl ether, mp 48–49°, $[\alpha]_D^{25°}$ 59.5 ± 2° (*c*, 2.2% in water).

Anal. Calcd for $C_8H_{15}NO_3$ (173.2): C, 55.5; H, 8.7; N, 8.1. Found: C, 55.4; H, 8.6; N, 8.2.

*N-Chloroacetyl-*D*-norvaline Methyl Ester.* D-Norvaline was chloroacetylated as described for the acetyl derivatives. The oily product was distilled, bp 115°/0.7 mm, $[\alpha]_D^{25°}$ 46.5 ± 1° (*c*, 1.5% in water).

Anal. Calcd for $C_8H_{14}ClNO_3$ (207.7): C, 46.3; H, 6.8; N, 6.7. Found: C, 46.1; H, 6.9; N, 6.6.

*Benzoyl-*D*-norvaline Methyl Ester.* D-Norvaline methyl ester was benzoylated as described for D-valine. The product was recrystallized from isopropyl ether–hexane, mp 76–77°, $[\alpha]_D^{25°}$ 20.8° ± 1.0° (*c*, 2.6% in MeOH).

Anal. Calcd for $C_{13}H_{17}NO_3$ (235.3): C, 66.4; H, 7.3; N, 5.9. Found: C, 66.5; H, 7.3; N, 5.9.

*Benzoyl-*L*-norvaline Methyl Ester.* L-Valine methyl ester was benzoylated as described for D-valine using an ether-water solvent mixture. The product was recrystallized from isopropyl ether–hexane, mp 76–77°, $[\alpha]^{25}$ −20.3 ± 1.0° (*c*, 3% in MeOH).

Anal. Calcd for $C_{13}H_{17}NO_3$ (235.3): C, 66.4; H, 7.3; N, 5.9. Found: C, 66.4; H, 7.3; N, 6.0.

Kinetic Studies. The kinetic studies were carried out as described previously by Applewhite *et al.* (1958). Values of K_I were obtained from the relation

$$K_I = \frac{v_0 K_0[I]}{k_0[E][S] - v_0(K_0 + [S])} \quad (2)$$

where v_0 is the initial velocity in the presence of the inhibitor. For the uninhibited hydrolysis of acetyl-L-leucine methyl ester, the values of K_0 and k_0 were taken as 3.76 ± 0.19 mM and 4.98 ± 0.14 sec^{-1}, respectively (Jones *et al.*, 1962).

TABLE I: Inhibition of the α-Chymotrypsin-catalyzed Hydrolysis of Acetyl-L-leucine Methyl Ester.

Inhibitor	[E][a] (μM)	[S][b] (mM)	[I][c] (mM)	K_I[d] (mM)	Runs[e]
Acetyl-D-valine methyl ester	3.456	1.39–6.97	69.4	>195.0	6–0
Chloroacetyl-D-valine methyl ester	3.456	1.39–6.97	32.4	169.0 ±55.0	5–0
Benzoyl-D-valine methyl ester	3.456	1.39–6.97	1.16	5.23 ± 2.07	5–0
Acetyl-D-norvaline methyl ester	3.456	1.34–4.02	39.6	111.0 ±37.0	5–0
Chloroacetyl-D-norvaline methyl ester	3.456	1.34–4.02	30.3	58.6 ± 4.5	5–0
Benzoyl-D-norvaline methyl ester	3.456	0.67–3.37	1.16	1.77 ± 0.39	5–0

[a] Based on a molecular weight of 25,000 and a nitrogen content of 16.5% for α-chymotrypsin. [b] Concentration range of acetyl-L-leucine methyl ester used as substrate in these determinations. [c] Concentration of inhibitor employed in each run. [d] The substrate activity of all inhibitors evaluated was negligible at the concentrations studied, and at enzyme concentrations up to 345.6 μM. [e] First number denotes number of experiments carried out to determine K_I, second number gives number of experiments rejected after application of a statistical reiterative procedure (Abrash *et al.*, 1960).

Results

Inhibition constants for the five compounds studied are given in Table I, along with details of the procedure. All compounds were tested as substrates of the enzyme, but none showed any evidence of α-chymotrypsin-catalyzed hydrolysis at enzyme concentrations up to 3.45 × 10^{-4} M. For all compounds K_I values were determined on the basis of competitive inhibition. This is justified (Webb, 1963), since in each case k_0 for the inhibited reaction is within experimental error of the value for the uninhibited reaction.

The chymotrypsin-catalyzed hydrolysis of *N*-benzoyl-L-norvaline methyl ester was determined in aqueous solutions at 25.0°, *p*H 7.90 ± 0.05, and 0.10 M in sodium chloride. A statistical reiterative procedure (Abrash *et al.*, 1960) was applied to ten experiments performed over a concentration range of 0.375–3.00 × 10^{-3} M to yield kinetic constants of K_0 = 0.85 ± 0.16 × 10^{-3} M and k_0 = 2.45 ± 0.14 sec^{-1}.

Table II compares the K_I values for D derivatives with K_0 and k_0 obtained previously for the L enantiomers. In every case, K_0 for the L isomer is less than K_I for the D isomer. The same probably holds true for *N*-chloroacetyl-D- and -L-norvaline methyl esters. An estimated value of K_0 for the L isomer, based on the regular sequence of K_0 values for the other three series examined, suggests that $K_0 \cong 5$ mM.

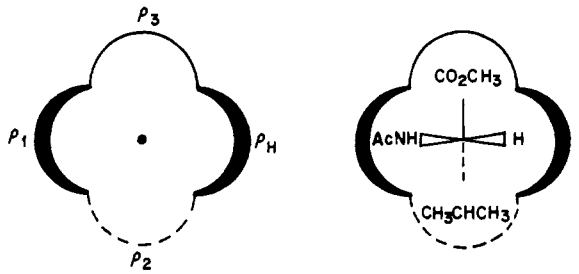

FIGURE 1: Schematic representation of the active site of α-chymotrypsin and its interaction with *N*-acetyl-L-valine methyl ester. The three-dimensional character is indicated by shadings: heavy lines indicate space in front of the plane of the figure, dotted lines indicate space behind this plane.

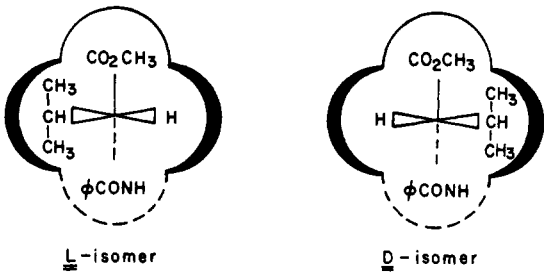

FIGURE 2: Schematic representation of the interaction of *N*-benzoylvaline methyl esters with the active site of α-chymotrypsin.

TABLE II: A Comparison of the Binding and Inhibition Constants of Several Acylated α-Amino Acid Methyl Esters.

Methyl Esters	K_0(L) (mM)	K_I(D) (mM)	K_0(L)/ K_I(D)
Acetylvaline	112.0[a]	>195.0	<0.57
Chloroacetylvaline	43.0[a]	169.0	0.25
Benzoylvaline	4.6[a]	5.2	0.88
Acetylnorvaline	10.2[b]	111.0	0.09
Chloroacetylnorvaline	~5.0[c]	58.6	~0.1
Benzoylnorvaline	0.85[d]	1.77	0.48
Acetylphenylalanine	1.8[e]	2.3	0.77

[a] Waite and Niemann (1962). [b] Jones and Niemann (1962). [c] Estimated; see text under Results for method used. [d] J. B. Jones and C. Niemann, unpublished experiment. [e] Hein and Niemann (1962).

Discussion

From a study of the interaction of acylated-L-valine methyl esters with α-chymotrypsin, Waite and Niemann (1962) concluded that *N*-acetyl-L-valine methyl ester interacted with the active site of the enzyme primarily to form a "productive" complex (Hein and Niemann, 1962; Niemann, 1964). If the active site of the enzyme is pictured as composed of four subgroups, ρ_1, ρ_2, ρ_3, and ρ_H, arranged so that they may interact with a substrate of general formula, $R_1'NHCHR_2$-COR_3, then a productive complex is defined as one that involves R_1—ρ_1, R_2—ρ_2, and R_3—ρ_3 interaction (Figure 1). In the case of *N*-benzoyl-L-alanine methyl ester, the drastically lower values obtained for both K_0 and k_0 led to the conclusion that the benzoyl derivative was bound predominantly in a "wrong-way" or nonproductive manner. The most likely possibility involved R_1—ρ_2 and R_3—ρ_1 interaction as the most significant. Similar reasoning suggested that, for the chloroacetyl derivative, K_0 was determined by a productive binding mode, as in the case of the acetyl derivative. The high values of K_0 for the *N*-acetyl and *N*-chloroacetyl compounds suggested that although none of the groups could bind particularly well to the enzyme, all three reactive groups, R_1, R_2, and R_3, contributed to determine the magnitude of K_0.

At present, it is not known whether K_0 values for a series of valine methyl esters represent binding constants, are proportional to binding constants, or vary with structural modifications as kinetic factors influence the magnitude of the observed values. The tetradentate model and the concept of productive and nonproductive binding modes predict certain relationships between K_0 values for L isomers and K_I values for the corresponding D antipodes. The data can be evaluated against this model.

If, for any given tetradentate compound, three specific interactions with the enzyme surface are necessary to determine K_0, then it is predicted that the K_0 value for its optical isomer, which cannot achieve the same configuration (Ogston, 1948), will be different. On the other hand, if the value of K_0 is determined primarily by the interaction of one or two groups with the enzyme, then it might be expected that D and L isomers would have similar K_0 values. If K_0 values are determined by kinetic parameters, one would not expect the same relationships.

The results for the D isomers of the acylated valine methyl esters bear out the predictions stated in terms of previous postulates (Hein and Niemann, 1962). If it is assumed that for the benzoyl derivative K_0 is determined primarily by the effectiveness of R_1—ρ_2 interaction and partially by R_3—ρ_3 interaction, then both D and L isomers can assume orientations relative to the asymmetric site which permit binding in this way (Figure 2). The observed ratio $K_{0L}/K_{ID} = 0.88$ indicates that the strength of binding is approximately the same for both compounds. For the case of the acetyl derivative $K_{0L}/K_{ID} < 0.57$ and for the chloroacetyl derivative the ratio is 0.25. If the L isomers of these compounds are bound predominantly, as indicated in Figure 1, then it is impossible for the D isomer to achieve the same interactions. As has been observed in many previous cases, the K_{0L}/K_{ID} ratios are less than u ity (Hein and Niemann, 1962).

Comparison of kinetic constants of the acylated norvaline derivatives suggests some further conclusions about the active site of α-chymotrypsin. Unlike the branched-chain valine compounds, norvaline derivatives are probably relatively successful in binding to the enzyme (Jones and Niemann, 1962). The *n*-propyl group can interact effectively with the ρ_2 locus (Jones *et al.*, 1965), resulting in a K_0 value one magnitude lower and a k_0 value more than one magnitude higher than that for the corresponding valine derivative. The more effective interaction of the *n*-propyl group with the enzyme surface makes it less likely that introduction of a bulky substituent in the R_1 position will lead to "wrong-way" binding.

As expected, the evidence for such unproductive binding modes is not clear. First, although the K_0 value for *N*-benzoyl-L-norvaline methyl ester is one magnitude lower than the K_0 value for *N*-acetyl-L-norvaline methyl ester, k_0 values for the two compounds are almost identical, 2.45 ± 0.14 and 2.70 ± 0.17 sec^{-1}, respectively. Second, the ratio of $(K_0)_L/(K_I)_D$ for benzoylnorvaline methyl ester is significantly different from 1.

The equivalence of observed catalytic rate constants for *N*-acetyl- and *N*-benzoyl-L-norvaline methyl esters indicates that the observed difference in K_0 is at least in part a measure of a difference in binding to the enzyme. This difference can best be attributed to more effective R_1—ρ_1 interaction. The deviation of the ratio of K_{0L}/K_{ID} from unity for the *N*-benzoylnorvaline methyl esters suggests the same conclusion. Similar values of K_{0L} and K_{ID} are not expected when binding constants are determined by the interaction of three groups with the enzyme surface.

In any case the explanation attributes a specific positive contribution to binding energy from R_1—ρ_1 interactions. While it has been recognized that R_1—ρ_1 interaction can profoundly modify the magnitude of k_0 (Hein and Niemann, 1962), the effect of R_1—ρ_1 interaction on K_0 for esters has been considered minimal. The present evidence indicates the possible efficacy of this interaction in determining the magnitude of K_0.

The results with the *N*-acetyl- and *N*-chloroacetyl-D-norvaline methyl esters also support the view that the nature of R_1 can affect K_0. For these compounds there is no reason to assume nonproductive binding modes, and the low ratios of K_{0L}/K_{ID} suggest that all three R—ρ interactions determine K_0. The low ratio, K_{0L}/K_{ID} = 0.09, illustrates the particular contribution of R_1—ρ_1 interaction in cases when R_2—ρ_2 interaction has not approached a limit situation.

For each of the acylated methyl esters studied, the lower K_0 and K_I values show that the norvaline compound binds much more strongly with the active site than does the corresponding valine derivative. As mentioned previously (Jones and Niemann, 1962), this effect may be attributed to the steric interaction with the protein surface of the isopropyl R_2 group of the valine series, possibly with the ρ_2 locus in the $S^{3E}_{R_2R_3}$ limiting cases, and with ρ_1 or ρ_H in the other limiting case.

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