if it competes with the substrate for the acetyl enzyme without completely blocking deacetylation. To overcome the substrate inhibition entirely, as in class A behavior, the inhibitor must have no effect on the deacetylation rate when bound by the acetyl enzyme.

CONCLUSION

The postulated mechanism for cholinesterase-catalyzed hydrolysis is in agreement with the experimental relationships between the optimum substrate concentration, the optimum velocity, and the inhibitor concentration. The mechanism explains many previously reported experiments (Augustinsson, 1948) in which diverse types of behavior were observed with different inhibitors, different substrates, and different experimental conditions, and with enzymes from different sources (Fig. 5, Table I). It also explains the small noncompetitive component observed with type II inhibitors such as tetramethylammonium. It is concluded that when the type II inhibitors become bound to the acetyl enzyme they at most partially block deacetylation. In contrast, deacetylation is prevented when the type I inhibitors, such as *cis* - 2 - dimethylaminocyclohexanol, become bound to the acetyl enzyme.

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REFERENCES

Augustinsson, K.-B. (1948), *Acta Physiol. Scand. 15*, suppl. 52.

Friess, S. L., and McCarville, W. J. (1954), *J. Am. Chem. Soc. 76*, 1363.

Krupka, R. M., and Laidler, K. J. (1961), *J. Am. Chem. Soc. 83*, 1445, 1448, 1454, 1458.

Main, A. R. (1961), *Biochem. J. 79*, 246.

Wilson, I. B. (1960), *in* The Enzymes, Boyer, P. S., Lardy, H., and Myrbäck, K., editors, New York, Academic Press, Inc., p. 501.

Wilson, I. B., and Alexander, J. (1962), *J. Biol. Chem. 237*, 1323.

The α-Chymotrypsin Catalyzed Hydrolysis of a Series of Acylated Glycine Methyl Esters[*]

JOHN P. WOLF, III, AND CARL NIEMANN[†]

From the Gates and Crellin Laboratories of Chemistry,
California Institute of Technology, Pasadena, California

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The kinetics of the α-chymotropsin catalyzed hydrolysis of twenty-two acylated glycine methyl esters in aqueous solutions at 25.0°, *p*H 7.90, and 0.50 M with respect to sodium chloride have been investigated. These data and those for the only previously known substrate of this class, methyl hippurate, have been integrated into a theory developed earlier to explain the structural and stereochemical specificity of α-chymotrypsin for trifunctional substrates of this enzyme.

In 1952 Huang and Niemann reported that the hydrolysis of methyl hippurate was catalyzed by α-chymotrypsin and through inhibition studies provided support for their conclusion that this substrate was hydrolyzed at the same site involved in the hydrolysis of previously recognized substrates of this enzyme. They also proposed that methyl hippurate be regarded as a bifunctional substrate to distinguish it from analogous trifunctional substrates which contained a side-chain as an additional structural feature. This distinction soon received experimental support from the more extended inhibition studies of Huang and Niemann (1953) and from those of Applewhite *et al.* (1958), Applewhite and Niemann (1959), and Hein and Niemann (1962). In these investigations it was found that methyl hippurate could be distinguished from representative trifunctional substrates by the character of the inhibition produced, for example, by indole. With trifunctional substrates the inhibition was fully competitive whereas with the bifunctional substrate it was of the mixed type (Dixon and Webb, 1958). The division of acylated α-amino acid derivatives into the two preceding classes of substrates has recently received further support from the studies of Wallace,[1] who found that whereas systems containing α-chymotrypsin and trifunctional substrates are relatively insensitive to the presence of 9-aminoacridinium ion, those containing a bifunctional substrate are substantially activated.

It is possible to distinguish substrates of any particular class on the basis of differences in the dependence of reactivity upon the structure of the substrate. Although an impressive amount of information of this kind is available for trifunctional substrates (Hein and Niemann, 1961), this is not the case for bifunctional substrates. To remedy this situation we have studied the α-chymotrypsin catalyzed hydrolysis of twenty-two acylated glycine methyl esters. It is the purpose of this communication to describe these experiments, to present further evidence for the distinction between bi- and trifunctional substrates, and to interpret the kinetic behavior of these bifunctional substrates in terms of the theory of the stereo- and structural specificity of α-chymotrypsin developed by Hein and Niemann (1961, 1962) from consideration of the behavior of the analogous trifunctional substrates.

EXPERIMENTAL

The twenty-two substrates were prepared as described below. All melting points were corrected and all analyses were by Dr. A. Elek.

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[†] To whom inquiries regarding this article should be sent.

[1] Wallace, R. A. (1962), unpublished results obtained in these laboratories.

Glycine Methyl Ester Hydrochloride.—This compound was prepared in 95% yield from glycine, methanol, and thionyl chloride by the method of Brenner and Huber (1953).

Acetylglycine Methyl Ester.—Acetylglycine (Herbst and Shemin, 1943) was esterified with methanol and thionyl chloride (Brenner and Huber, 1953). The crude product was distilled at 88° and 0.15 mm Hg and the distillate, which crystallized on standing, was twice recrystallized from diisopropyl ether to give 80% of acetylglycine methyl ester, m.p. 57.0–58.7°.

Anal. Calcd. for $C_5H_9O_3N$ (131): C, 45.8; H, 6.9; N, 10.7. Found: C, 45.7; H, 7.0; N, 10.6.

Propionylglycine Methyl Ester.—Glycine methyl ester hydrochloride, 40 g, was dissolved in 75 ml of distilled water saturated with sodium chloride and sodium carbonate. To this solution was added 150 ml of toluene, and the mixture was homogenized with a Vibro-stirrer and maintained at 0° in an ice-salt bath. A solution of 18.7 g of propionyl chloride in toluene made up to 100 ml with this solvent was added in 10-ml portions to the preceding homogenized mixture. Solid sodium carbonate was added as needed to keep the reaction mixture alkaline. The mixture was stirred for 15 minutes after the addition of the last portion of acid chloride and transferred to a 500-ml separatory funnel, and the toluene phase was collected and the aqueous phase extracted three times with 100-ml portions of ethyl acetate. The toluene and ethyl acetate extracts were combined, the solvents were removed by vacuum distillation, and the residual oil, 13 g, was distilled at 110° and 1.5 mm. The distillate crystallized on standing and was recrystallized from diisopropyl ether to give 55% of propionylglycine methyl ester, long needles m.p. 37.0–38.0°.

Anal. Calcd. for $C_6H_{11}O_3N$ (145): C, 49.6; H, 7.6; N, 9.6. Found: C, 49.4; H, 7.4; N, 9.5.

Isobutyrylglycine Methyl Ester.—A solution of 18 g of isobutyric acid and 9 g of thionyl chloride in 60 ml of toluene was refluxed for one hour, and the solution was cooled and made up to 100 ml with toluene. This solution was used to acylate glycine methyl ester hydrochloride by the procedure described immediately above. The crude product, a light oil, was distilled at 90° and 0.3 mm, and the distillate was allowed to crystallize and the solid recrystallized from diisopropyl ether to give 51% of isobutyrylglycine methyl ester, long needles, m.p. 50.8–51.6°.

Anal. Calcd. for $C_7H_{13}O_3N$ (159): C, 52.8; H, 8.2; N, 8.8. Found: C, 52.9; H, 8.3; N, 8.7.

Pivalylglycine Methyl Ester.—Pivalyl chloride was obtained by slowly distilling a solution of 1 mole of pivalic acid dissolved in 3 moles of benzoyl chloride as described by Brown (1938). The acid chloride was used to acetylate glycine methyl ester hydrochloride by the procedure described above for the preparation of propionylglycine methyl ester. The crude pivalylglycine methyl ester was distilled at 111–112° and 4 mm to give a distillate which crystallized after standing at 4° for 5 months but which could not be recrystallized except from the melt. The distilled product, n_D^{25} = 1.4492, was obtained in 80% yield.

Anal. Calcd. for $C_8H_{15}O_3N$ (173): C, 55.4; H, 8.7; N, 8.1. Found: C, 55.3; H, 8.6; N, 8.2.

Isovalerylglycine Methyl Ester.—This compound was prepared from isovaleric acid and glycine methyl ester hydrochloride as described for the analogous isobutyrylglycine methyl ester. The crude product was twice distilled at 96.5° and 0.3 mm. The final distillate, n_D^{25} = 1.4481, resisted crystallization.

Anal. Calcd. for $C_8H_{15}O_3N$ (173): C, 55.4; H, 8.7; N, 8.1. Found: C, 55.4; H, 8.7; N, 8.0.

t-Butylacetylglycine Methyl Ester.—*t*-Butylacetyl chloride was prepared in 74% yield from 50 g of *t*-butylacetic acid and 140 g of benzoyl chloride (Brown, 1938). Reaction of 43 g of the acid chloride with 40 g of glycine methyl ester hydrochloride as described for the preparation of propionylglycine methyl ester gave a crude oily product which was distilled at 107° and 0.5 mm to give a clear viscous liquid which crystallized on standing overnight at 4°. The final product was a solid, m.p. 43.5–44°.

Anal. Calcd. for $C_9H_{17}O_3N$ (187): C, 57.7; H, 9.2; N, 7.5. Found: C, 57.7; H, 9.1; N, 7.5.

Chloroacetylglycine Methyl Ester.—Chloroacetic acid was converted into the acid chloride by the procedure of Brown (1938). Glycine methyl ester hydrochloride was acylated with chloroacetyl chloride as described for the preparation of propionylglycine methyl ester. The crude product was distilled at 105° and 0.5 mm to give a distillate which crystallized on standing. Recrystallization of this product from diisopropyl ether gave chloroacetylglycine methyl ester, m.p. 43.6–44.6°.

Anal. Calcd. for $C_5H_8O_3NCl$ (166): C, 36.3; H, 4.8; N, 8.5. Found: C, 36.4; H, 4.9; N, 8.5.

Dichloroacetylglycine Methyl Ester.—Substitution of dichloroacetic acid for chloroacetic acid in the procedure described immediately above gave a crude product in 50% yield. Recrystallization of this material three times from diisopropyl ether gave dichloroacetylglycine methyl ester, m.p. 63.1–64.5°.

Anal. Calcd. for $C_5H_7O_3NCl_2$ (200): C, 30.0; H, 3.5; N, 7.0. Found: C, 30.2; H, 3.6; N, 6.9.

Trichloroacetylglycine Methyl Ester.—Substitution of trichloroacetic acid for chloroacetic acid in the procedure described above gave a crude product in 80% yield. Distillation of this preparation at 95.6° and 0.2 mm gave trichloroacetylglycine methyl ester, a viscous oil, n_D^{25} = 1.4953.

Anal. Calcd. for $C_5H_6O_3NCl_3$ (234): C, 25.6; H, 2.6; N, 6.0. Found: C, 25.5; H, 2.5; N, 5.9.

Trifluoroacetylglycine Methyl Ester.—Glycine methyl ester hydrochloride (15 g) was acylated with 25 g of trifluoroacetic anhydride in the presence of triethylamine by the procedure described below for the preparation of picolinylglycine methyl ester. The crude product was first distilled at 49–50° and 0.15 mm and then redistilled at 68.0–68.5° and 1.1 mm to give the desired product as a colorless liquid.

Anal. Calcd. for $C_5H_6O_3NF_3$ (185): C, 32.5; H, 3.3; N, 7.6. Found: C, 32.6; H, 3.5; N, 7.4.

Ethoxyacetylglycine Methyl Ester.—Ethoxyacetic acid was prepared from chloroacetic acid and sodium ethoxide as described by Rothstein (1932). The acid was converted to the acid chloride by the procedure of Brown (1938) and the latter compound used to acylate glycine methyl ester hydrochloride as described for the preparation of propionylglycine methyl ester. The crude product was distilled at 97–100° and 0.3 mm to give 80% of product, a viscous oil, n_D^{25} = 1.4481.

Anal. Calcd. for $C_7H_{13}O_4N$ (175): C, 48.0; H, 7.5; N, 8.0. Found: C, 48.2; H, 7.3; N, 7.9.

Ethylmercaptoacetylglycine Methyl Ester.—Ethylmercaptoacetic acid was prepared from chloroacetic acid and sodium ethyl mercaptide by the procedure of Ramberg (1907). The acid chloride was prepared as before (Brown, 1938) and used to acylate glycine methyl ester hydrochloride as in the preceding preparation. The crude product was twice recrystallized from diisopropyl ether to give the ester, m.p. 60.7–62.7°, in 80% yield.

Anal. Calcd. for $C_7H_{13}O_3NS$ (191): C, 44.0; H, 6.8; N, 7.3. Found: C, 44.0; H, 6.8; N, 7.4.

Carboethoxyglycine Methyl Ester.—Glycine methyl ester hydrochloride was acylated with ethyl chloroformate as described for the preparation of propionylglycine methyl ester. The crude product was an oil which was distilled at 73.1° and 0.25 mm to give a colorless liquid, n_D^{25} = 1.4367, in 60% yield.

Anal. Calcd. for $C_6H_{11}O_4N$ (161): C, 44.7; H, 6.9; N, 8.7. Found: C, 44.8; H, 6.8; N, 8.6.

Methanesulfonylglycine Methyl Ester. — Glycine methyl ester hydrochloride was acylated with methanesulfonyl chloride as described for the preparation of propionylglycine methyl ester. The crude product obtained in 90% yield was distilled at 125° and 0.35 mm to give a distillate which crystallized on standing to yield a solid product, m.p. 32.0°.

Anal. Calcd. for $C_4H_9O_4NS$ (167): C, 28.7; H, 5.4; N, 8.4. Found: C, 28.7; H, 5.3; N, 8.2.

Succinylglycine Methyl Ester.—Succinylglycine was prepared by heating an equimolar mixture of glycine and succinic anhydride in an oil bath at 190° for 3 hours. The reaction product was cooled, powdered, and esterified with methanol and thionyl chloride (Brenner and Huber, 1953). The crude ester obtained in 60% yield was recrystallized from a mixture of ethyl acetate and diisopropyl ether to give the final product, m.p. 93.6–95.4°.

Anal. Calcd. for $C_7H_9O_4N$ (171): C, 49.1; H, 5.3; N, 8.2. Found: C, 49.1; H, 5.2; N, 8.3.

Phenylacetylglycine Methyl Ester.—Glycine methyl ester hydrochloride was acylated with phenylacetyl chloride as in the preparation of propionylglycine methyl ester. The crude ester, obtained in 60% yield, was recrystallized from diisopropyl ether to give the final product, long needles, m.p. 89.3–90.8°.

Anal. Calcd. for $C_{11}H_{13}O_3N$ (207): C, 63.7; H, 6.3; N, 6.8. Found: C, 63.6; H, 6.2; N, 6.9.

p - Aminobenzoylglycine Methyl Ester.—*p* - Aminohippuric acid was esterified with methanol and thionyl chloride (Brenner and Huber, 1953) to give the crude ester in 75% yield. Recrystallization from ethyl acetate gave *p*-aminobenzoylglycine methyl ester, short needles, m.p. 131.9–133.1°.

Anal. Calcd. for $C_{10}H_{12}O_3N_2$ (208): C, 57.7; H, 5.8; N, 13.5. Found: C, 57.6; H, 5.8; N, 13.4.

Picolinylglycine Methyl Ester.—A mixture of picolinic acid, excess thionyl chloride, and sufficient toluene to effect solution was refluxed for 24 hours. The solution, containing *ca.* 0.2 equivalents of the acid chloride, was made up to 100 ml with toluene. Glycine methyl ester hydrochloride, 0.3 equivalents, was suspended in anhydrous chloroform, 0.66 equivalents of triethylamine was added, and the reaction mixture was cooled to 0° in an ice-salt bath. The toluene solution of the acid chloride was then added, in 10-ml portions, to the chloroform solution of the ester, with shaking and cooling after each addition. After all the acid chloride had been added the reaction mixture was allowed to stand at 25° for 1 hour, then transferred to a 500-ml separatory funnel, and the non-aqueous phase was washed three times with a dilute aqueous sodium bicarbonate solution that had been saturated with sodium chloride. The aqueous washings were discarded and the solvents removed from the nonaqueous phase by vacuum distillation. The residue was an oil, which was twice distilled at 123° and 0.2 mm to give a 20% yield of picolinylglycine methyl ester as a colorless liquid.

Anal. Calcd. for $C_9H_{10}O_3N_2$ (194): C, 55.7; H, 5.2; N, 14.4. Found: C, 55.5; H, 5.2; N, 14.3.

Nicotinylglycine Methyl Ester.—Nicotinyl chloride was prepared from sodium nicotinate and thionyl chloride (Meyer and Graf, 1928). The toluene solution containing *ca.* 0.2 equivalents of the acid chloride was filtered and made up to 100 ml with toluene. This solution was then used to acylate glycine methyl ester hydrochloride by the procedure used immediately above for the preparation of picolinylglycine methyl ester. In the present case the crude acylated ester crystallized on standing. It was recrystallized from diisopropyl ether to give the final product, m.p. 69.9–71.7°, in 80% yield.

Anal. Calcd. for $C_9H_{10}O_3N_2$ (194): C, 55.7; H, 5.2; N, 14.4. Found: C, 55.6; H, 5.2; N, 14.3.

Isonicotinylglycine Methyl Ester.—The acid chloride of isonicotinic acid was obtained by refluxing the acid in excess thionyl chloride for 18 hours in a manner similar to that used for the preparation of picolinyl chloride. In one instance 200 ml of dry hexane was added to the reaction mixture to precipitate a brown microcrystalline product. When an attempt was made to distill this substance *in vacuo* thionyl chloride was evolved, leaving a black tar. Further attempts to purify the precipitate were abandoned, and a toluene solution of the crude acid chloride was used to acylate glycine methyl ester hydrochloride as described for the preparation of picolinylglycine methyl ester. The final product, m.p. 112.0–113.5°, after recrystallization from diisopropyl ether, was obtained in 60% yield.

Anal. Calcd. for $C_9H_{10}O_3N_2$ (194): C, 55.7; H, 5.2; N, 14.4. Found: C, 55.7; H, 5.2; N, 14.5.

α-Furoylglycine Methyl Ester.—Glycine methyl ester hydrochloride was acylated with α-furoyl chloride using the procedure employed for the preparation of propionylglycine methyl ester. The crude product, obtained in 75% yield, was twice recrystallized from diisopropyl ether to give the final product, long slender needles, m.p. 70.1–70.9°.

Anal. Calcd. for $C_8H_9O_4N$ (183): C, 52.5; H, 4.9; N, 7.7. Found: C, 52.6; H, 4.9; N, 7.7.

β-Indolylglycine Methyl Ester.—Glycine methyl ester hydrochloride was acylated with crude β-indolyl chloride (Peterson *et al.*, 1958) by the procedure previously employed for the preparation of picolinyl-, nicotinyl-, and isonicotinylglycine methyl esters. The crude product was obtained in a 30% yield based upon indole. Recrystallization of the crude product from a mixture of toluene and ethyl acetate gave the acylated ester, blunt needles, m.p. 169–171°.

Anal. Calcd. for $C_{12}H_{12}O_2N_2$ (232): C, 62.1; H, 5.2; N, 12.1. Found: C, 61.9; H. 5.3; N, 12.1.

Kinetic Studies.—All enzyme-catalyzed reactions were conducted in aqueous solutions at 25.0 ± 0.1°, *p*H 7.90 ± 0.02, and 0.50 M with respect to sodium chloride. The procedure, involving use of a *p*H-stat (Nielands and Cannon, 1955), was identical with that described by Applewhite *et al.* (1958). Considerable attention was devoted to the question of the enzyme blank reaction. On the basis of studies which will be reported elsewhere, it was concluded that for the substrates considered in this communication, which in every case gave linear $d[P]/dt$ recorder traces at the concentrations specified, no correction for the enzyme blank reaction was required. In contrast, all initial velocities for the enzyme catalyzed reactions were corrected for the base (hydroxyl ion) catalyzed substrate blank reactions. In order to make this correction, which in many cases was substantial, k_B, the constant for the base-catalyzed hydrolysis of each substrate, was evaluated over a *p*H range from 7.90 to 9.00 and for a range of substrate concentrations comparable to those employed in the corresponding enzymatic hydrolyses. For these concentrations and with $[OH^-]$ assumed to be given by the *p*H of the system, the base-catalyzed hydrolysis was first order in both substrate and hydroxyl ion concentration. The constant k_B was computed from a least squares fit to the relation $v = k_B[S][OH^-]$. In practice, for each experiment at a particular substrate concentration, the velocity of the base-catalyzed hydrolysis, computed from the previously determined value of k_B and the pertinent base and substrate concentrations, was subtracted from the value of the initial velocity evaluated for the total system from the slope of the linear $d[P]/dt$ recorder trace. The corrected initial velocities and the corresponding initial values of $[S]$ and $[E]$ were then fitted to the equation $v_0 = -K_0v_0/[S] + k_0[E]$ by the method of least squares. The actual computations were performed on a Datatron 205 digital computer essentially as described by Abrash *et al.* (1960).

When it was found that four of the compounds initially selected for study were too unreactive to permit evaluation of their kinetic constants, three, pivalylglycine methyl ester, succinylglycine methyl ester, and trichloroacetylglycine methyl ester, were examined with respect to their ability to function as inhibitors of the α-chymotrypsin catalyzed hydrolysis of nicotinylglycine methyl ester. These studies were conducted essentially as described above with $[S] = 5.0$ to 30.0 mM and with $[I] = 14.1, 23.4$, and 8.76 mM respectively. With the first two compounds no inhibition could be detected. However, with trichloroacetylglycine methyl ester the inhibition appeared to be substantially competitive, $k_0 = 0.482$ and 0.527 sec.$^{-1}$ for the uninhibited and inhibited reactions, respectively, with $K_I = 5.30$ mM.

An Armour preparation of crystalline, bovine, salt free α-chymotrypsin, lot No. 283, was used in all experiments.

TABLE I

α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYLGLYCINE METHYL ESTER[a]

Enzyme-Catalyzed Hydrolysis[b]				Base-Catalyzed Hydrolysis			
Expt.	$[S]_0$[c]	v_t[d]	v[e]	Expt.	$[S]_0$[c]	pH	v_t[f]
1	5.0	5.19	4.52	1	10.0	7.90	1.55
2	10.0	7.93	6.65	2	10.0	8.50	5.41
3	15.0	10.57	8.65	3	25.0	8.50	13.27
4	20.0	12.40	9.85	4	15.0	9.00	23.42
5	25.0	15.42	12.23	5	20.0	9.00	32.81
6	30.0	17.11	13.28				

[a] In aqueous solutions at 25.0 ± 0.1°, pH 7.90 ± 0.02 and 0.50 M with respect to sodium chloride. [b] With $[E] = 4.16 \times 10^{-5}$ M based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for α-chymotrypsin. [c] In units of 10^{-3} M. [d] Uncorrected initial velocity in units of M min.$^{-1} \times 10^{-6}$. [e] Corrected initial velocity in units of M min.$^{-1} \times 10^{-6}$. [f] Velocity of base-catalyzed hydrolysis in units of M min.$^{-1} \times 10^{-6}$.

RESULTS

A representative protocol is given in Table I and all results are summarized in Table II. Where it was possible to determine the reaction kinetics, the rates of all enzyme catalyzed reactions could be described by equation (1). In no case was the maximum extent of

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

reaction more than 10%. Therefore, there was no reason to anticipate inhibition of the reaction by its reaction products (Braunholtz *et al.*, 1959).

For sixteen of the twenty-two acylated glycine methyl esters examined as substrates of α-chymotrypsin, it was possible to evaluate the constants k_0 and K_0 of equation (1). Thus, there are available for consideration values of these two constants for seventeen acylated glycine methyl esters if one includes the constants for the previously investigated methyl hippurate. In addition, there are four acylated glycine methyl esters which do not appear to be substrates and two which are substrates but where the individual constants were not evaluated because of experimental difficulties.

DISCUSSION

One theory of the specificity of α-chymotrypsin for trifunctional substrates (Hein and Niemann, 1961, 1962) is based upon the proposition that a given substrate may combine with the active site of the enzyme in a number of alternative modes, some of which are non-productive and fully competitive with the productive mode or modes. For a trifunctional substrate of the general formula $R_1'CONHCHR_2COR_3$, where $R_1'CONH = R_1$ and $R_2 \neq H$, the problem of relating kinetic data and structure requires evaluation of the relative contributions made to both orientation and binding of the substrate at the active site by interaction of the structural components R_1, R_2, and COR_3 with the loci ρ_1, ρ_2, ρ_3 and ρ_H at this site. It is not sufficient to consider only the interaction of R_1 with ρ_1, R_2 with ρ_2 and COR_3 with ρ_3. Rather, one must include all possible combinations of these interactions, *e.g.*, R_1 with ρ_1, ρ_2, ρ_3, and ρ_H, R_2 with ρ_1, ρ_2, ρ_3, and ρ_H, *etc.* It is then possible from a limited number of *a priori* and empirical postulates to deduce which of the possible modes of combination is most favored. Thus, one can predict which modes and interactions will determine the magnitude of the constant K_0 and which will control that of the constant k_0. In a series of methyl esters the latter is determined by the effect of the various interactions on the orientation of the carbomethoxy group with respect to attacking nucleophilic and/or electrophilic groups of the active site. As an enzyme provides an intramolecular environment for a reaction that otherwise would be intermolecular, the rate of decomposition of the intermediate enzyme-substrate complex is dependent upon the degree to which a favorable geometry between the attacked and attacking groups can be achieved. The stability of the reactive complex is represented by K_0 only in the absence of non-productive modes of combination.

The solution of the preceding problem for trifunctional substrates (Hein and Niemann, 1961, 1962) is in principle easier than for bifunctional substrates of the same enzyme. The difficulty in the latter case arises from the greater number of degrees of freedom accessible to bifunctional substrates in their combination with the active site of the enzyme, a feature which makes it more difficult to separate and evaluate the various individual interactions. However, by drawing upon information obtained with the trifunctional substrates, it has been possible to make substantial progress in interpreting the kinetic behavior of bifunctional substrates of the general formula $R_1'CONHCH_2COR_3$.

We begin by calling attention to the plot of k_0 *vs.* K_0 given in Figure 1, which includes all values of these two constants available from Table II. It is at once evident that, just as in the case of trifunctional substrates, there is no simple relationship between values of k_0 and K_0 despite the fact that all of the data were obtained for a series of acylated glycine methyl esters. Thus, we may reject the idea that the kinetic properties of these substrates can be explained on the basis of a single simple relationship between stability of the enzyme-substrate complex and rate of formation of products. On the positive side, the data given in Figure 1 lead us to believe that values of K_0 can be regarded as apparent dissociation constants, since

TABLE II

α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF A SERIES OF ACYLATED GLYCINE METHYL ESTERS[a]

R_1' of $R_1'CONHCH_2CO_2CH_3$	$[S]_0$ (mM)	K_0 (mM)	k_0[b] (sec.$^{-1}$)	k_0/K_0 (M^{-1} sec.$^{-1}$)	k_B (M^{-1} sec.$^{-1}$)
CH_3	5.0–30.0	30.7 ± 5.9	0.013 ± 0.002	0.437	2.66
C_2H_5	5.0–30.0	38.0 ± 6.2	0.018 ± 0.003	0.471	2.32
$(CH_3)_2CH$	5.0–30.0	45.4 ± 4.5	0.021 ± 0.002	0.471	1.95
$(CH_3)_3C$	5.2–25.8		too unreactive to evaluate		1.49
$(CH_3)_2CHCH_2$	5.1–30.6	24.0 ± 1.6	0.025 ± 0.002	1.02	1.33
$(CH_3)_3CCH_2$[c]	39.8		too unreactive to evaluate		—
$ClCH_2$	5.0–30.0	68.7 ± 1.0	0.055 ± 0.001	0.803	3.73
Cl_2CH	5.0–30.0	21.8 ± 1.4	0.023 ± 0.001	1.06	3.95
Cl_3C	5.1–25.5		too unreactive to evaluate		3.83
F_3C	5.0–30.0	53.1 ± 5.0	0.085 ± 0.005	1.59	6.92
$C_2H_5OCH_2$	5.1–30.8		first order in $[S]$	0.159	5.03
$C_2H_5SCH_2$	5.0–30.0	10.4 ± 0.1	0.0054 ± 0.0002	0.519	2.13
C_2H_5O	5.3–31.6	65 ± 12	0.131 ± 0.021	2.01	2.12
CH_3SO_2[d]	5.2–311.6	85 ± 10	0.012 ± 0.008	0.140	5.27
$C_2H_4(CO)_2$[e]	7.9–47.5		too unreactive to evaluate		6.15
$C_6H_5CH_2$	2.5–15.0	7.85 ± 0.50	0.006 ± 0.001	0.777	2.50
C_6H_5[f]		7.55 ± 0.30	0.200 ± 0.003	26.49	2.94[g]
p-H_2N-C_6H_4	2.5–15.0	8.29 ± 0.09	0.041 ± 0.001	4.89	2.02
α-C_5H_4N[h]	5.06		too reactive to evaluate[i]		
β-C_5H_4N[j]	10.0–50.0	31.3 ± 0.4	0.460 ± 0.005	14.69	2.10
γ-C_5H_4N[k]	5.0–150.0	17.7 ± 0.4	0.159 ± 0.001	8.97	2.98
α-C_4H_3O[l]	5.0–30.0	19.6 ± 0.7	0.969 ± 0.028	49.46	2.77
β-C_8H_6N[m]	0.7–4.13	1.37 ± 0.14	0.056 ± 0.003	40.65	2.10

[a] In aqueous solutions at 25.0 ± 0.1°, pH 7.90 ± 0.02, and 0.50 M with respect to sodium chloride with $[E] = 3.85$–4.17×10^{-5} M unless otherwise noted. [b] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for α-chymotrypsin. [c] $[E] = 3.51 \times 10^{-5}$ M. [d] General formula does not apply, compound is $CH_3SO_2NHCH_2CO_2CH_3$. [e] General formula does not apply, compound is succinylglycine methyl ester. [f] Determined by Applewhite *et al.* (1958). [g] Determined by Gordon (1959). [h] α-Pyridyl. [i] Also with $[E] \simeq 4 \times 10^{-6}$ M. [j] β-Pyridyl. [k] γ-Pyridyl. [l] α- or 2-Furyl. [m] β- or 3-Indolyl.

there is no evidence that values of K_0 are determined by those of k_0.

In order to relate the interpretation of the kinetic data for bifunctional substrates to that for trifunctional substrates, it is instructive to compare the results for the simplest members of each series, acetylglycine methyl ester, $K_0 = 30.7$ mM, $k_0 = 0.013$ sec.$^{-1}$, $k_0/K_0 = 0.437$ M^{-1} sec.$^{-1}$, and acetyl-L-alanine methyl ester, $K_0 = 611$ mM, $k_0 = 1.29$ sec.$^{-1}$, $k_0/K_0 = 2.11$ M^{-1} sec.$^{-1}$ (Wolf, 1959). The latter is one of the less reactive trifunctional acylated α-amino acid ester type substrates. Its combination with the active site of α-chymotrypsin may be envisaged as involving a significant COR_3–ρ_3 interaction, a marginal R_2–ρ_2 interaction, and an effective R_1–ρ_1 interaction, with all interactions being important for orientation of the substrate at the active site and probably all three determining the magnitude of the enzyme-substrate dissociation constant (Hein and Niemann, 1961, 1962). In assessing the role of R_2 in the over-all process, it should be noted that for trifunctional substrates an increase in the size of R_2 generally leads to a decrease in the magnitude of K_0 and an increase in that of k_0 (Hein and Niemann, 1961, 1962; Jones and Niemann, 1962).

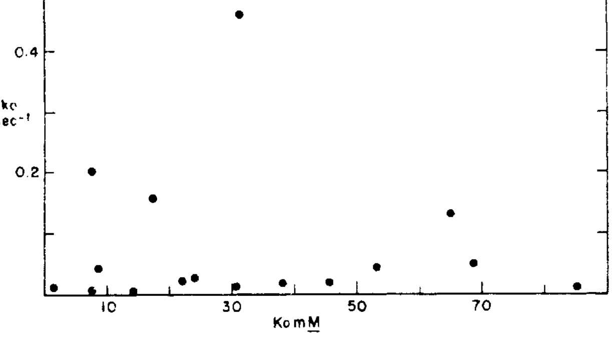

FIG. 1.—Absence of a direct relation between values of k_0 and K_0 for seventeen bifunctional substrates of α-chymotrypsin.

Replacement of the α-methyl group of acetyl-L-alanine methyl ester by hydrogen, with its concomitant loss of asymmetry, leads to a decrease in the magnitude of K_0, a change in the opposite direction from that expected on the basis of the behavior of analogous trifunctional substrates. That the value of k_0 is decreased by almost two orders of magnitude is not unexpected, at least for the direction of the change. However, the concomitant decrease in both constants suggests the application of a principle used previously to interpret the kinetic behavior of certain trifunctional substrates (Hein and Niemann, 1962). We conclude that the constant K_0, which we assume to be a dissociation constant, or the quotient of the appropriate combination of several such constants, is being determined by a mode or modes of combination other than that leading to an intermediate in the formation of products. The data suggest that the dissociation constant of the non-productive complex is probably between one and two orders of magnitude smaller than the dissociation constant of the productive complex for this bifunctional substrate.

The first six compounds listed in Table II are members of a set of acylated glycine methyl esters in which the structure of the acyl component has been varied systematically. There are two prominent features of this set. One, all values of k_0/K_0 are less than the corresponding values of k_B. Two, there is less than a two-fold variation in the four determinable values of k_0.

Since the ratio k_0/K_0 has the same units as k_B, a direct comparison of the rates of enzyme and base (hydroxide) catalyzed reactions can be made. Because the values of k_0/K_0 for all six substrates of the first set are less than those of k_B, we are led to the conclusion that on a mole basis α-chymotrypsin is a less effective catalyst for these substrates than sodium hydroxide. There are two possible explanations of this behavior. Either the substrates under consideration are not being hydrolyzed at the active site involved in the hydrolysis of trifunctional substrates of this enzyme, or both bi- and trifunctional substrates interact at the same site but the former combine in such a way that the characteristic features of enzyme catalysis, as judged by the behavior of the latter, are largely lost. We tentatively choose the second alternative largely because it conforms with Occam's razor (Northrop, 1961). The higher rates of formation of reaction products which we associate with precise orientation of the substrate at the active site are absent, but the ability of the substrate to combine with the enzyme is still present. This behavior is consistent with a predominance of non-productive modes of combination.

Further evidence for the significance of alternative, non-productive binding modes, as well as a suggestion as to the nature of this binding, is supplied by examining the least reactive members of the first six acylated glycine methyl esters listed in Table II. Both pivalyl and *t*-butylacetylglycine methyl esters are too unreactive to be evaluated as substrates. When an attempt was made to evaluate the former compound as an inhibitor of the α-chymotrypsin catalyzed hydrolysis of nicotinylglycine methyl ester, no inhibition could be detected with $[I] = 14.1$ mM. From this experiment we infer that K_I for pivalylglycine methyl ester is greater than 100 mM. The behavior of the pivalyl and *t*-butylacetylglycine methyl esters suggests that the lack of reactivity of these compounds as substrates of α-chymotrypsin is due primarily to a substantial increase in the dissociation constants of their respective productive enzyme-substrate complexes. Similar behavior, to a more limited degree, is noted for the series acetyl, propionyl, and isobutyrylglycine methyl ester where an increase in the value of K_0 is accompanied by a more modest increase in that of k_0. On the other hand, for isovalerylglycine methyl ester the value of K_0 is decreased relative to that of acetylglycine methyl ester again without any drastic alteration in the value of k_0.

The fact that K_0 values can be increased significantly without much change in those of k_0 is evidence that at least the lower K_0 values are primarily determined by non-productive binding modes. A comparison with trifunctional substrates which are presumably bound more frequently in productive modes supports the above conclusion. α-*N*-Pivalyl-L-tyrosinhydrazide, with a higher K_0 value and a lower k_0 value, is less reactive than α-*N*-acetyl-L-tyrosinhydrazide (Lutwack *et al.*, 1957). Acetyl-L-valine methyl ester, with a much higher K_0 value and a much lower k_0 value, is considerably less reactive than acetyl-L-norvaline methyl ester (Jones and Niemann, 1962).

The behavior of the two pairs of trifunctional substrates indicates the nature of the non-productive binding encountered with the glycine derivatives. An increase in the value of K_0 may result from steric factors influencing interactions with the ρ_2 site. These same factors may also cause a decrease in k_0 (Jones and Niemann, 1962). The data for the glycine derivatives may be interpreted by assuming that the non-productive binding mode involves R_1–ρ_2 interaction.

Isovalerylglycine methyl ester does not follow the preceding pattern, and it is not obvious why this structure leads to an anomalous situation. With the added knowledge that α-methyl-*n*-butyrylglycine methyl ester is a very poor substrate of α-chymotrypsin,[2] we believe it premature to attempt a detailed interpretation of the kinetic behavior of the group of compounds where $R_1' = C_4H_9$, particularly in the absence of information about the properties of *n*-butyryl- and *n*-valerylglycine methyl ester. However, we can assert that bifunctional substrates of the type $R_1'CONHCH_2CO_2CH_3$, where R_1' is an alkyl group, are characterized by a very low order of reactivity associated with a high incidence of non-productive combination, and the absence of orienting factors leading to even a modest reactivity of the productive complexes. They provide a dramatic example of the importance of an α-amino acid side-chain in enhancing the probability of formation of productive enzyme-substrate complexes and of their facile decomposition into reaction products. The surprising feature is the facility with which certain of these compounds, *e.g.*, where R_1' is methyl or isobutyl, combine with the enzyme to form principally non-productive complexes.

Comparison of acetylglycine methyl ester, $K_0 = 30.7$ mM, $k_0 = 0.013$ sec.$^{-1}$, $k_0/K_0 = 0.437$ M^{-1} sec.$^{-1}$, with chloroacetylglycine methyl ester, $K_0 = 68.7$ mM, $k_0 = 0.055$ sec.$^{-1}$, $k_0/K_0 = 0.803$ M^{-1} sec.$^{-1}$, the first of the next four compounds listed in Table II, reveals that the greater reactivity of the latter substrate is achieved by more than a 4 fold increase in the magnitude of k_0 which more than compensates for the 2.2 fold increase in the magnitude of K_0. These facts suggest that replacement of one of the methyl hydrogen atoms present in the acylamino component of acetylglycine methyl ester by a chlorine atom diminishes the degree of non-productive combination and probably enhances orientation of the substrate in the productive complex, both factors operating in the same direction to cause an increase in the value of k_0. As before, we assume that the dissociation constant of the productive enzyme-substrate complex is substantially greater than the value of K_0.

It has been found that for acetyl-L-valine methyl ester both K_0 and k_0 are substantially greater than the corresponding values for chloroacetyl-L-valine methyl ester (Waite and Niemann, 1962). Thus, the same replacement in a trifunctional substrate as that performed on an analogous bifunctional substrate leads to exactly the opposite result. This behavior is explicable when it is recalled that for the above trifunctional substrates the R_1–ρ_1 interaction is adequate for orientation but both the R_2–ρ_2 and COR_3–ρ_3 interactions are marginal, the former because of the relatively small size of the R_2 component and the latter because of steric shielding of the COR_3 function (Hein and Niemann, 1961, 1962; Jones and Niemann, 1962). Nevertheless, the predominant conformation determining K_0 is that associated with productive combination. With this situation perturbation of the R_1–ρ_1 interaction could readily change the conformation of the substrate at the active site and alter the degree of non-productive combination. In the case of the glycine derivative the decreased significance of the R_1–ρ_2 interaction is coupled with the greater orienting ability of the R_1–ρ_1 interaction to produce a more reactive substrate than acetylglycine methyl ester.

The data obtained for chloroacetylglycine methyl ester, $K_0 = 68.7$ mM, $k_0 = 0.055$ sec.$^{-1}$, $k_0/K_0 = 0.803$ M^{-1} sec.$^{-1}$; dichloroacetylglycine methyl ester, $K_0 = 21.8$ mM, $k_0 = 0.023$ sec.$^{-1}$, $k_0/K_0 = 1.06$ M^{-1} sec.$^{-1}$; and trichloroacetylglycine methyl ester, $K_I = 5.3$ mM, are

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[2] Hein, G. E., and Wallace, R. A. (1962), unpublished experiments conducted in these laboratories.

particularly informative. They provide evidence that values of K_0 are indeed apparent dissociation constants, that bifunctional substrates of the type $R_1'CONHCH_2CO_2CH_3$ can combine with α-chymotrypsin at the site occupied by trifunctional substrates of this enzyme, that competition between productive and non-productive modes of combination is a real and not an imaginary phenomenon and that successive replacement of the methyl hydrogen atoms of the acylamino component of acetylglycine methyl ester by chlorine leads to a set of compounds which are increasingly effective in combining with the active site of the enzyme.

The fact that trichloroacetylglycine methyl ester is a competitive inhibitor of the α-chymotrypsin catalyzed hydrolysis of nicotinylglycine methyl ester, taken with the knowledge that numerous simple pyridine derivatives are competitive inhibitors of the hydrolysis of acetyl-L-valine methyl ester by the same enzyme,[1] allows us to conclude that the above bi- and trifunctional compounds interact with the enzyme at a common site. The extrapolation of this conclusion to include all bifunctional substrates considered in this communication is reasonable in that all are acylated glycine methyl esters. This does not mean that all are similarly oriented at the active site.

The consistent decline in the magnitude of values of k_0 and K_0 in the series chloro-, dichloro-, and trichloroacetylglycine methyl ester terminating with K_0 determinable only as K_I and k_0 so small as to be indeterminable is readily interpreted in terms of the formation of fully competitive non-productive complexes with lower dissociation constants than those of the productive complexes. In fact, it is one of the better examples of this phenomenon largely because it was possible through a systematic change in structure to reach the limit where all combination was essentially non-productive.

It will be recalled that successive replacement of the methyl hydrogen atoms of the acylamino component of acetylglycine methyl ester by methyl groups results in a modest increase in the values of k_0 and K_0 until two of the hydrogen atoms have been replaced. Beyond this point a compound is produced which no longer has demonstrable ability to combine with the enzyme and hence is unable to function either as a substrate or inhibitor. In this instance it is reasonable to conclude that replacement of one or two of the hydrogen atoms by methyl groups diminishes the extent of non-productive combination without markedly influencing that of productive combination. However, replacement of all three hydrogen atoms by methyl groups limits both productive and non-productive combination because of the intrusion of steric factors. In contrast, replacement of the same hydrogen atoms by chlorine atoms clearly leads to quite different results. Thus, pivalylglycine methyl ester is unreactive because it does not combine with the enzyme. Trichloroacetylglycine methyl ester is not a substrate because combination although effective is totally unproductive. The latter compound is the most effective known aliphatic competitive inhibitor of α-chymotrypsin.

The behavior of the fourth member of the second set, trifluoroacetylglycine methyl ester, K_0 = 53.1 mM, k_0 = 0.085 sec.$^{-1}$, k_0/K_0 = 1.59 M^{-1} sec.$^{-1}$, is best viewed in relation to that of acetyl- and trichloroacetylglycine methyl ester. Whereas replacement of the three methyl hydrogen atoms of the acylamino component of acetylglycine methyl ester by chlorine leads to an effective competitive inhibitor, replacement by fluorine leads to the most reactive substrate of the first and second set. However, relative to even the poor trifunctional substrates its reactivity is not impressive. Here again, sodium hydroxide is a better catalyst than α-chymotrypsin. The greater reactivity of trifluoroacetylglycine methyl ester relative to acetylglycine methyl ester appears to arise from a decrease in the extent of non-productive complex formation associated with the ability of the trifluoroacetamido group to function more effectively than an acetamido group in orienting the substrate to give a productive complex rather than from increased intrinsic susceptibility of the ester function to hydrolysis. The dramatic difference in the behavior of trifluoroacetyl- and trichloroacetylglycine methyl ester could arise if electrical polarizability were a significant factor in binding of the latter compound to its respective loci at the active site.

The third set of compounds listed in Table II, *i.e.*, ethoxyacetyl-, ethylmercaptoacetyl-, carboethoxy-, methanesulfonyl-, and succinylglycine methyl ester, provide kinetic data that are difficult to interpret principally because the set is structurally heterogenous and supplementary data needed to provide a clue to the nature of the dominant interactions are lacking. For the present, the information obtained is useful only in suggesting that substrates of the type $ROCONHCH_2CO_2R'$ are worthy of further attention.

The remaining eight compounds listed in Table II contain an aromatic component. The behavior of phenylacetylglycine methyl ester, K_0 = 7.85 mM, k_0 = 0.006 sec.$^{-1}$, k_0/K_0 = 0.777 M^{-1} sec.$^{-1}$, is best compared to that of methyl hippurate, K_0 = 7.55 mM, k_0 = 0.200 sec.$^{-1}$, k_0/K_0 = 26.49 M^{-1} sec.$^{-1}$. In this case insertion of a methylene group between the benzene nucleus and the carboxyl group of the acylamino component has essentially no effect upon the value of K_0 but causes a decrease in that of k_0 of more than 30 fold. There is little question that for these two compounds the values of K_0 are for all practical purposes the dissociation constants of the non-productive enzyme-substrate complexes. The fact that the two K_0 values are nearly identical and also of the order of 7.5 mM is consistent with the observation that practically all simple monosubstituted benzenoid compounds that do not bear a formal charge have K_I values of 7.5 ± 3.5 mM.[1] For these latter compounds the magnitude of the inhibition constant, K_I, is relatively insensitive to the nature of the substituent. It is probable, but by no means certain, that, when simple uncharged benzenoid compounds combine with the active site of α-chymotrypsin to form unproductive complexes whose dissociation constants are of the order of 7.5 mM, they do so by interaction with the ρ_2 locus of the active site.

The kinetic constants for acetyl- and benzoyl-L-alanine methyl ester are, K_0 = 611 mM and k_0 = 1.29 sec.$^{-1}$ (Wolf, 1959), and K_0 = 9.75 mM and 0.261 sec.$^{-1}$ (Hein and Niemann, 1961, 1962) respectively. Also, $K_0 = K_{S\cdot 1}K_{S\cdot 2}/(K_{S\cdot 1} + K_{S\cdot 2})$ and $k_0 = k_2K_{S\cdot 2}/(K_{S\cdot 1} + K_{S\cdot 2})$, where $K_{S\cdot 1}$ and $K_{S\cdot 2}$ are the dissociation constants of the respective productive and non-productive enzyme-substrate complexes, and k_2 is the rate of decomposition of the productive complex (Hein and Niemann, 1961). These facts and the values of K_0 and k_0 for phenylacetyl- and benzoylglycine methyl ester suggest that the values of $K_{S\cdot 1}$ for these substrates are significantly greater than 7.5 mM and that $K_{S\cdot 1}$ for phenylacetylglycine methyl ester is very much greater than that for benzoylglycine methyl ester. If productive combination of these substrates with the active site involves R_1–ρ_1 and COR_3–ρ_3 interactions we may conclude that the R_1–ρ_1 interaction for benzoylglycine is only moderately effective and that for phenylacetylglycine methyl ester is very poor, in fact so poor that there is little effective orientation of the carbomethoxy group at the COR_3 locus. In the latter case k_2 is close to the lower limit permitting recognition of an enzyme-catalyzed reaction.

Turning to the pair *p*-aminobenzoyl- and benzoylglycine methyl ester, K_0 = 8.29 and 7.55 mM and k_0 = 0.041 and 0.200 sec.$^{-1}$, respectively, it is seen that replacement of a *p*-hydrogen atom by an amino group has relatively little effect upon the dissociation constant of the non-productive complex. Its effect is to markedly diminish the extent of productive complex formation by *p*-aminobenzoylglycine methyl ester relative to benzoylglycine methyl ester as judged by the 5 fold decrease in value of k_0 accompanying this structural modification.

In the two preceding paragraphs we have been concerned with structural modifications that lead to a decrease in the extent of productive combination. Fortunately, there are several examples of the opposite effect, *i.e.*, where an increase in the extent of productive combination can be achieved by suitable choice of the acylamino component. The problem is to select an acylamino component whose affinity for the ρ_2 locus is not overwhelming and which can interact with the ρ_1 locus in a positive manner. In a sense the replacement of acetyl by chloroacetyl in the case of acetylglycine methyl ester was a step in this direction. However, in this instance the outcome although in the right direction was not notably successful.

The knowledge that pyridine is a competitive inhibitor of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-valine methyl ester and has a K_I value of *ca.* 30 mM (Huang and Niemann, 1953; footnote 1, this paper) led us to examine the series isonicotinyl-, nicotinyl-, and picolinylglycine methyl ester. For isonicotinylglycine methyl ester K_0 = 17.7 mM and k_0 = 0.159 sec.$^{-1}$. Thus, with a hetero atom containing an unshared pair of electrons *para* to the carbonyl group of the acyl component, the isonicotinyl derivative exhibits a behavior comparable in direction but not in magnitude to that observed for the *p*-aminobenzoyl derivative. However, for nicotinylglycine methyl ester K_0 = 31.3 mM and k_0 = 0.460 sec.$^{-1}$. In this instance positioning of the hetero atom in the *meta* rather than the *para* position is associated with an almost 3 fold increase in the magnitude of k_0 and to a point where it is more than double that observed for benzoylglycine methyl ester. The extension of the preceding tactic to picolinylglycine methyl ester, with the hetero atom in the *ortho* position, was in a sense too successful. The latter compound is so reactive as a substrate of α-chymotrypsin that we were unable to evaluate its kinetic constants even at an enzyme concentration of *ca.* 10^{-6} M. In the absence of such values, a deficiency which we are now attempting to rectify by working at still lower enzyme concentrations, we can predict that when these constants are determined the value of k_0 will be found to be considerably greater than that of nicotinylglycine methyl ester. Prediction of the value of K_0 for picolinylglycine methyl ester is more difficult. From the trend in values of K_0 for isonicotinyl- and nicotinylglycine methyl ester one might expect a value of K_0 for picolinylglycine methyl ester greater than that observed for the nicotinyl derivative. However, this trend may be deceptive, since it is not known which of the several dissociation constants is determining the value of K_0 for nicotinylglycine methyl ester.

The preceding results suggest that productive combination is improved, probably through a more effective R_1–ρ_1 interaction, when the acylamino component contains a hetero atom containing an unshared pair of electrons bonded to the carbon atom α to its carbonyl group. This suggestion led to the examination of α-furoylglycine methyl ester to determine whether oxygen could replace nitrogen as the hetero atom. The kinetic constants obtained for this substrate, K_0 = 19.6 mM and k_0 = 0.969 sec.$^{-1}$, not only answer this question in the affirmative but in the absence of numerical data for picolinylglycine methyl ester demonstrate that it is possible for simple bifunctional substrates of the type $RCONHCH_2CO_2CH_3$ to attain a reactivity comparable to that of the poorer ester type trifunctional substrates. It will be recalled that, for acetyl-L-alanine methyl ester, K_0 = 611 mM and k_0 = 1.29 sec.$^{-1}$. With α-furoylglycine methyl ester it is possible to get within 75% of the latter value of k_0 without benefit of a side-chain to assist in orientation of the substrate in the productive enzyme-substrate complex. It is expected that β-furoylglycine methyl ester will be markedly less reactive.

Picolinyl- and α-furoylglycine methyl ester are superior to methyl hippurate as model bifunctional substrates of α-chymotrypsin. Our experience with these compounds leads us to predict that certain acylated glycylglycine methyl esters, such as acetylglycylglycine methyl ester, will have k_0 values substantially greater than those of the corresponding acylated glycine methyl esters. Substrates of the type $RCO(NHCHR'CO)_nNHCH_2CO_2CH_3$ also are excellent candidates for the further exploration of the ρ_1 locus, provided R and R′ are chosen to minimize interaction with the ρ_2 locus. Our attempt to enter this latter area with α-methyl-*n*-butyrylglycine methyl ester was defeated by the inertness of the compound as a substrate.

The last compound to be considered, β-indolylglycine methyl ester, K_0 = 1.37 mM, k_0 = 0.056 sec.$^{-1}$, k_0/K_0 = 40.65 M^{-1} sec.$^{-1}$, provides a final example of effective combination but relatively little in the form of a productive complex. Other evidence (Hein and Niemann, 1961, 1962) leads us to believe that the indole nucleus in the absence of overriding orientation factors tends to interact with the ρ_1 locus. In the case at hand the relatively low value of k_0 probably arises because the carbomethoxy group in β-indolylglycine methyl ester is too distant by one atom from the indole nucleus to provide for effective placement of the carbomethoxy group in the enzyme-substrate complex. β-Indolylglycine methyl ester may be viewed not as an analog but as the next higher homolog of tryptophan methyl ester insofar as relative position of indole nucleus and carbomethoxy group is concerned. In this respect the former compound may be compared to phenylacetylglycine methyl ester, which is far less reactive than methyl hippurate for presumably similar reasons.

In the preceding discussion we have been able to place twenty-three bifunctional compounds of the type $R_1'CONHCH_2CO_2CH_3$ within the framework of a theory of the structural and stereochemical specificity of α-chymotrypsin developed for trifunctional substrates of this enzyme. In general, the treatment for the bifunctional substrates has been less quantitative than achieved earlier for the trifunctional substrates. This is largely a result of the fact that relatively few of the bifunctional substrates represented reasonable approaches to limit cases. This was expected because of the greater number of degrees of freedom accessible to the bifunctional compounds when combining with the active site of the enzyme. However, with the discovery of several more reactive bifunctional substrates a new approach to possible limit cases has been disclosed. Thus, one can anticipate that future studies along the lines suggested in this communication will result in a more quantitative treatment of the bifunctional substrate of the type $R_1'CONHCH_2CO_2R_3$.

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REFERENCES

Abrash, H. I., Kurtz, A. N., and Niemann, C. (1960), *Biochim. Biophys. Acta 45,* 378.
Applewhite, T. H., Martin, R. B., and Niemann, C. (1958), *J. Am. Chem. Soc. 80,* 1457.
Applewhite, T. H., and Niemann, C. (1959), *J. Am. Chem. Soc. 81,* 2208.
Braunholtz, J. T., Kerr, R. J., and Niemann, C. (1959), *J. Am. Chem. Soc. 81,* 2852.
Brenner, M., and Huber, W. (1953), *Helv. Chim. Acta 36,* 1109.
Brown, H. C. (1938), *J. Am. Chem. Soc. 60,* 1325.
Dixon, M., and Webb, E. C. (1958), Enzymes, New York, Academic Press, Inc., pp. 172–179.
Gordon, T. P. (1959), Ph.D. Thesis, California Institute of Technology, Pasadena, Calif.
Hein, G. E., and Niemann, C. (1961), *Proc. Nat. Acad. Sci. U. S. 47,* 1341.
Hein, G. E., and Niemann, C. (1962), *J. Am. Chem. Soc.* in press.
Herbst, R. M., and Shemin, D. (1943), *Org. Syn.,* Coll. Vol. II, p. 11.
Huang, H. T., and Niemann, C. (1952), *J. Am. Chem. Soc. 74,* 4634.
Huang, H. T., and Niemann, C. (1953), *J. Am. Chem. Soc. 75,* 1395.
Jones, J. B., and Niemann, C. (1962), *Biochemistry 1,* 1093.
Lutwack, R., Mower, H. F., and Niemann, C. (1957), *J. Am. Chem. Soc. 79,* 5690.
Meyer, H., and Graf, R. (1928), *Ber. 61,* 2202.
Nielands, J. B., and Cannon, M. D. (1955), *Anal. Chem. 27,* 29.
Northrop, J. H. (1961), *Ann. Rev. Biochem. 30,* 1.
Peterson, P. E., Wolf, J. P., III, and Niemann, C. (1958), *J. Org. Chem. 23,* 303.
Ramberg, L. (1907), *Ber. 40,* 2588.
Rothstein, B. (1932), *Bull. Soc. Chim. 51,* 838.
Waite, H., and Niemann, C. (1962), *Biochemistry 1,* 250.
Wolf, J. P., III (1959), Ph.D. Thesis, California Institute of Technology, Pasadena, Calif.

Effect of Acetylation on the Active Site of Several Antihapten Antibodies: Further Evidence for the Presence of Tyrosine in Each Site*

A. L. GROSSBERG AND D. PRESSMAN

From the Department of Biochemistry Research, Roswell Park Memorial Institute,
New York State Department of Health, Buffalo, New York

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Extensive modification of four antihapten antibodies with acetic anhydride resulted in loss of binding activity in each case. The presence of hapten during acetylation partially prevented these losses. This observation indicated that a group in the site of each antibody was being attacked. Reversal of the losses of binding activity by treatment of the acetylated antibodies with hydroxylamine indicated that a hydroxyl group had been acetylated in each site. In view of this fact and other data previously reported, it was concluded that a tyrosine residue is present in each of the antibody sites. Of the antibodies studied (anti-*p*-azobenzenearsonate [anti-R_p], anti-*p*-azobenzoate [anti-X_p], anti-*p*-azophenyltrimethylammonium [anti-A_p], and anti-3-azopyridine [anti-P_3]) only anti-R_p and anti-X_p appeared to lose activity by partial acetylation of amino groups in the molecule. Of these, only the former appeared to have an amino group in the site. Acetylation of mixtures of two antibodies and analysis of complete binding curves of modified antibodies allowed determination of the relative sensitivity of the antibodies to acetylation and of the relative effects of acetylation on blocking binding sites and on alteration of binding constants. It could be concluded that the importance for binding of the acetylatable group varied for the several antibodies. The sensitivity toward acetylation was in the order, anti-R_p $\overline{>}$ anti-X_p $\gg$ anti-A_p $>$ anti-P_3.

We have been studying the nature of antibody sites by determining the effect of chemical modification on specific binding activity. Whether the effect is due to reaction of a group in the binding site itself rather than elsewhere in the molecule can be determined by carrying out the alteration in the presence and in the absence of the specific hapten. If the hapten protects against the loss of sites which is caused by alteration in the absence of hapten, then the loss must have been due to reaction of a group in the site.

Recently we compared four different antihapten antibodies with respect to the effect of iodination on binding activity (Grossberg *et al.,* 1962). We studied antibodies to haptens bearing a positive charge, a negative charge, and no charge, to give a broad range of different antibody types. The antibodies studied were anti-*p*-azobenzoate (anti - X_p), anti - *p* - azobenzenearsonate (anti - R_p), anti - *p* - azophenyltrimethylammonium (anti-A_p), and anti - 3 - azopyridine (anti - P_3). Iodination of each of these was found to reduce antibody activity, and it was concluded that each of the antibodies contained an iodinatable residue, probably tyrosine, in the antibody site.

Nisonoff and Pressman (1959) reported that extensive acetylation of anti-X_p antibody with acetic anhydride resulted in loss of antibody sites. We are now reporting the effect of acetylation with acetic anhydride on the antibody activity of all four of the above antihapten antibodies. Antibody activity is lost when each anti-

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