The α-Chymotrypsin-catalyzed Hydrolysis of α-*N* and *O*-Alkyl Derivatives of α-*N*-Acetyl-L-tyrosine Methyl Ester*

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An investigation of the kinetics of the α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetyl-α-*N*-methyl-L-tyrosine methyl ester and of α-*N*-acetyl-*O*-methyl, -ethyl, and -isopropyl-L-tyrosine methyl ester, in aqueous solutions at 25.0°, *p*H 7.90, and 0.10 M in sodium chloride, has shown that the lesser reactivity of these substrates, relative to α-*N*-acetyl-L-tyrosine methyl ester, is not due to an inability of the modified molecules to combine with the active site of the enzyme. The diminished reactivity arises from a less favorable orientation of the modified molecules when present at the active site.

α-*N*-Acylated aromatic α-amino acid esters are among the more reactive substrates of α-chymotrypsin and are frequently employed in studies on the mechanism of action of this enzyme. However, there are many features of the structural specificity of α-chymotrypsin for substrates of this type that require elucidation. In this communication we shall concern ourselves with the kinetic consequences of (a) replacement of the α-amido hydrogen atom of α-*N*-acetyl-L-tyrosine methyl ester by a methyl group, and (b) alkylation of the phenolic hydroxyl group of the same substrate with a methyl, ethyl, or isopropyl group.

Experimental

N-p-Tosyl-L-tyrosine.—Esterification of 94.2 g of L-tyrosine with absolute ethanol and thionyl chloride (Brenner and Huber, 1953) followed by recrystallization of the crude product from a mixture of absolute ethanol and ligroin gave 114 g (89%) of L-tyrosine ethyl ester hydrochloride. To this product suspended in 800 ml of chloroform was added 25 g of sodium carbonate dissolved in a small amount of water. The mixture was shaken until all ester had dissolved in the chloroform phase, whereupon a chloroform solution of 95.3 g of *p*-toluenesulfonyl chloride was added in small portions and with vigorous shaking over a period of several hours to the biphasic reaction mixture. An aqueous solution of 30.8 g of sodium carbonate was then added and the mixture shaken for an additional 4 hours. The chloroform phase was separated and dried over anhydrous sodium sulfate, and the chloroform was removed by distillation *in vacuo* until the solution became turbid. The residual solution was heated to boiling, hot ligroin was added, and the solution was allowed to cool to give 158 g (94%) of product, mp 113–114.5°. Recrystallization of this product from a mixture of chloroform and ligroin gave *N-p*-tosyl-L-tyrosine ethyl ester, mp 113.5–114.5°, $[\alpha]_D^{25}$ 6.3 (*c*, 0.4% in absolute ethanol). The ester, 189.7 g, was saponified at 50° with 750 ml of 4 N aqueous sodium hydroxide. The reaction mixture was cooled in an ice bath and carefully acidified with concd hydrochloric acid to give 173 g (99%) of *N-p*-tosyl-L-tyrosine, mp 185–187°, $[\alpha]_D^{20}$ −22.9° (0.5 N aqueous sodium hydroxide). Recrystallization from 20% aqueous ethanol gave a product, mp 187.5–188°, $[\alpha]_D^{20}$ −23.3° (0.5 N aqueous sodium hydroxide). Fischer and Lipschitz (1915) report the following constants: mp 187–188°, $[\alpha]_D^{20}$ −8.58° (0.5 N aqueous sodium hydroxide).

N-p-Toysl-O,N-dimethyl-L-tyrosine.—To a solution of 29 g of *N-p*-tosyl-L-tyrosine in 175 ml of 3 N aqueous sodium hydroxide was added slowly and with stirring 33 ml of dimethyl sulfate. After all of the dimethyl sulfate had been added the reaction mixture was stirred at 55° for an additional 2 hours. The precipitated crystalline sodium salt of *N-p*-tosyl-*O,N*-dimethyl-L-tyrosine was collected and dried to give 26.2 g of crude product. Solid sodium hydroxide, 21 g, was added to the filtrate which was treated as before with 33 ml of dimethyl sulfate to give an additional 2.8 g of crude product. The two portions of sodium salt were combined and dissolved in hot water, the solution was filtered, and the filtrate was acidified with concd hydrochloric acid to give 23.5 g (75%) of the desired acid. Recrystallization from aqueous ethanol and from a mixture of ethyl acetate and igroin gave *N-p*-tosyl-*O,N*-dimethyl-L-tyrosine, mp 143.5–144°, $[\alpha]_D^{25}$ −26.0° (*c*, 3.64% in absolute ethanol).

Anal. Calcd. for $C_{18}H_{21}O_5NS$ (363): C, 59.5; H, 5.8. Found: C, 59.6; H, 5.9.

The preceding compound was also prepared by a modification of the procedure of Fischer and Lipschitz (1915) involving methylation with methyl iodide. This procedure was less convenient than that described and gave lower yields and a less pure product.

α-N-Methyl-L-tyrosine.—A solution of 5.0 g of *N-p*-tosyl-*O,N*-dimethyl-L-tyrosine in 50 ml of 55–58% redistilled hydriodic acid was refluxed for 10 hours under nitrogen. The dark red solution was poured into water, and the suspension was chilled and filtered to remove the precipitated *p*-thiocresol. The filtrate was extracted with ethyl ether and the pale yellow aqueous phase was evaporated to dryness. The residue was taken up in water and again evaporated to dryness. This procedure was repeated several times. After the final evaporation the crystalline residue was dissolved in a small volume of boiling water and the hot solution was neutralized with ammonium hydroxide. The precipitate so obtained was dissolved in hot 0.3 N aqueous hydrochloric acid and the colorless solution was again neutralized to give 2.36 g (88%) of α-*N*-methyl-L-tyrosine, mp 268–271°, with decomp, $[\alpha]_D^{25}$ 18.2° (*c*, 1.1% in 10% aqueous hydrochloric acid).

A modification of the above procedure in which 5.0 g of *N-p*-tosyl-*O,N*-dimethyl-L-tyrosine was refluxed with 25 ml of 55–58% redistilled hydriodic acid and 5 ml of 50% hypophosphorous acid for 2

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hours gave 2.5 g (93%) of α-*N*-methyl-L-tyrosine, mp 270.5–271.5°, with decomp, $[\alpha]_D^{25}$ 19.6° (*c*, 1.1% in 10% aqueous hydrochloric acid). This procedure appears to be superior to the first on the basis of both yield and purity of product.

The direct α-*N*-methylation of L-tyrosine (Corti, 1949) was also attempted. To a well-stirred solution of 100 g of L-tyrosine in 900 ml of N aqueous sodium hydroxide was added dropwise 80 ml of dimethyl sulfate. After all the latter reagent had been added the temperature of the reaction mixture was raised to 90° and kept there for 5 hours. The reaction mixture was cooled, and the colorless precipitate was collected and washed with ice water. The moist precipitate was added to 1.5 liters of boiling water, and after the addition of 23 ml of concd hydrochloric acid the solution was treated with Norit and filtered. From the cooled filtrate there was obtained 20 g (19%) of α-*N*-methyl-L-tyrosine, lustrous needles, mp 269°, with decomp, $[\alpha]_D^{25}$ 19.1° (*c*, 1.44% in 10% aqueous hydrochloric acid). Neutralization of the mother liquor gave an additional 4.3 g (4%) of product, $[\alpha]_D^{25}$ 19.1° (*c*, 1.4% in 10% aqueous hydrochloric acid) bringing the total yield to 23% which was substantially lower than that obtained (58%) by the less direct route.

α-N-Methyl-L-tyrosine Methyl Ester Hydrochloride.—Esterification of 22 g of α-*N*-methyl-L-tyrosine with methanol and thionyl chloride (Brenner and Huber, 1953) gave 27.1 g (98%) of crude ester hydrochloride which was recrystallized from a mixture of methanol and ligroin to give 25.8 g (93%) of α-*N*-methyl-L-tyrosine methyl ester hydrochloride, fine colorless needles, mp 143–144°.

α-N-Acetyl-α-N-methyl-L-tyrosine Methyl Ester.—To a cold aqueous solution of 7.38 g of α-*N*-methyl-L-tyrosine methyl ester hydrochloride was added 1.59 g of sodium carbonate dissolved in the minimum amount of water. The slightly basic solution was shaken with cold ethyl acetate. Successive portions of acetyl chloride in ethyl acetate and aqueous sodium carbonate were added to the biphasic reaction mixture until no detectable turbidity was observable upon the addition of a further portion of acetyl chloride. The ethyl acetate phase was collected, successively washed with dilute aqueous hydrochloric acid, aqueous sodium carbonate, and water, and dried over anhydrous sodium sulfate, and the solution was evaporated *in vacuo* until a crystalline precipitate began to form. The suspension was heated to effect solution and sufficient boiling ligroin was added to produce a distinct turbidity. From the cooled suspension there was obtained 7.4 g (98%) of crude product. Recrystallization of the crude product from a mixture of ethyl acetate and ligroin gave 6.8 g (90%) of α-*N*-acetyl-α-*N*-methyl-L-tyrosine methyl ester, long colorless needles, mp 132.5–133.0°, $[\alpha]_D^{25}$ − 81.5 ± 0.5° (*c*, 0.96% in pyridine).

Anal. Calcd. for $C_{13}H_{17}O_4N$ (251): C, 62.1; H, 6.8; N, 5.6. Found: C, 62.2; H, 6.8; N, 5.5.

α-N-Acetyl-O-methyl-L-tyrosine.—The following procedure was developed from several earlier ones (Karrer, *et al.*, 1922; du Vigneaud and Meyer, 1932; Lehr and Clarke, 1932; Synge, 1939). To a mechanically stirred suspension of 45 g of L-tyrosine in 750 ml of water maintained at 90° on a steam bath was added dropwise over a period of 45 minutes 200 ml of acetic anhydride. The solution was stirred at 90° for an additional hour and cooled, and a minuscule amount of unreacted L-tyrosine was removed by filtration. The slightly yellow filtrate was evaporated *in vacuo* to a yellow viscous oil. The oil was dissolved in aqueous acetone and again evaporated as before. This procedure was repeated two more times in order to eliminate all the acetic acid initially present. The resulting viscous yellow oil was dissolved in 150 ml of warm 5 N aqueous sodium hydroxide. To this solution was added dropwise with efficient stirring 41 ml of dimethyl sulfate. Toward the end of the addition it was necessary to add 50 ml of 2.5 N aqueous sodium hydroxide to maintain the *p*H of the reaction mixture above *p*H 10. The reaction mixture was heated to 75–80° for an additional 3 hours, cooled, and acidified to *p*H 2 with concd hydrochloric acid, whereupon a gummy precipitate separated. The addition of 500 ml of chloroform followed by stirring at 0° for 0.5 hour led to the formation of a granular precipitate. This precipitate was collected, washed with ethyl ether, and dried *in vacuo*. The biphasic filtrate was evaporated *in vacuo* until a substantial amount of a granular precipitate had separated. This precipitate was treated as before and combined with the first fraction to give a total of 46.5 g (79%) of crude product, mp 145–149°, $[\alpha]_D^{25}$ 53.2° (*c*, 1.2% in methanol). This product gave a faintly positive test with the Folin-Denis reagent (Folin, 1929; Folin and Ciocalteu, 1927; Herriott, 1935–36; Alcott and Fraenkel-Conrat, 1947). Recrystallization of the crude product from hot water gave α-*N*-acetyl-*O*-methyl-L-tyrosine, mp 149–150°, $[\alpha]_D^{25}$ 54.2° (*c*, 1.2% in methanol), negative test with the Folin-Denis reagent.

α-N-Acetyl-O-methyl-L-tyrosine Methyl Ester.—Esterification of 11.86 g of α-*N*-acetyl-*O*-methyl-L-tyrosine with 50 ml of methanol and 4.3 ml of thionyl chloride (Brenner and Huber, 1953) gave, after removal of excess methanol, a gummy product which was triturated with cold aqueous sodium bicarbonate to give a crystalline precipitate. The precipitate was collected, washed with ice water, and dried *in vacuo* to give 9.2 g (73%) of product, mp 103–105°, $[\alpha]_D^{25}$ 27.8° (*c*, 1.44% in methanol). The Folin-Denis test was negative. This product was taken up in toluene, decolorized with Norit, and allowed to crystallize from a mixture of toluene and ligroin to give α-*N*-acetyl-*O*-methyl-L-tyrosine methyl ester, fine needles, mp 104–105°, $[\alpha]_D^{25}$ 25.3 ± 1.4° (*c*, 0.35% in methanol).

Anal. Calcd. for $C_{13}H_{17}O_4N$ (251): C, 62.1; H, 6.8; N, 5.6. Found: C, 62.2; H, 6.8; N, 5.6.

α-N-Acetyl-O-ethyl-L-tyrosine.—Crude syrupy α-*N*-acetyl-L-tyrosine, prepared from 9 g of L-tyrosine, *vide ante*, was dissolved in 60 ml of warm 4 N aqueous potassium hydroxide. Fifty ml of ethanol and 20 ml of ethyl iodide were added to the pale yellow solution, and the biphasic system was emulsified with the aid of a Vibro-stirrer and maintained in this condition for 8 hours at 45°. Stirring was then stopped, the phases were separated, and the yellow aqueous phase was extracted with ethyl ether and diluted with 50 ml of water. It was then evaporated *in vacuo* to *ca.* 50 ml and acidified to *p*H 2 with concd hydrochloric acid. The oil which separated was triturated with chloroform to form a crystalline product. This product was collected, washed with ethyl ether, and recrystallized twice from boiling water to give 4.5 g of α-*N*-acetyl-*O*-ethyl-L-tyrosine, fine needles, mp 175.5–176.5°, $[\alpha]_D^{24}$ 52.2 ± 2.5° (*c*, 1.0% in methanol). The Folin-Denis test was negative. An additional 3.0 g of product, mp 176–176.5°, $[\alpha]_D$ 52.0 ± 2.5° (*c*, 1.0% in methanol) giving a negative Folin-Denis test was recovered from the various mother liquors. The total yield was 60%.

α-N-Acetyl-O-ethyl-L-tyrosine Methyl Ester.—α-*N*-Acetyl-*O*-ethyl-L-tyrosine was esterified as described for the *O*-methyl analog. In this instance two re-

TABLE I

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF α-*N*-METHYL AND OF *O*-METHYL, ETHYL, AND ISOPROPYL α-*N*-ACETYL-L-TYROSINE METHYL ESTER[a]

Derivative	No. Expts.[b]	[E] (μmoles)[c]	[S] mM	K_0[d] mM	k_0[d] (sec^{-1})	k_0/K_0 (M^{-1} sec^{-1})
N-Methyl	11, 3	100.4	0.53–4.2	8.4 ± 1.8	0.026 ± 0.004	3.1
O-Methyl	8, 0	1.48	0.78–6.25	2.6 ± 0.4	0.488 ± 0.029	186
O-Ethyl	8, 0	0.315	1.06–8.4	12.4 ± 2.1	10.2 ± 1.2	822
O-Isopropyl	8, 0	34.9	0.16–1.3	1.6 ± 0.4	0.012 ± 0.005	7.2

[a] In aqueous solutions at 25.0 ± 0.1°, *p*H 7.90 ± 0.01, and 0.10 M in sodium chloride. [b] Number of experiments performed for evaluation of K_0 and k_0; second number refers to those rejected by statistical reiterative evaluation procedure. [c] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5%. [d] Evaluated by a least squares fit to the equation $([E][S]/v_0) = (K_c/k_0) + ([S]/k_0)$ as described in text.

crystallizations from toluene gave the desired ester, mp 117–118°, $[\alpha]_D$ 29.3 ± 0.4 (*c*, 1.2% in methanol).

Anal. Calcd. for $C_{14}H_{19}O_4N$ (265): C, 63.4; H, 7.2; N, 5.3. Found: C, 63.4; H, 7.2; N, 5.2.

α-N-Acetyl-O-isopropyl-L-tyrosine.—Syrupy α-*N*-acetyl-L-tyrosine, prepared from 11.0 g of L-tyrosine and 53 ml of acetic anhydride, *vide ante*, was dissolved in 72 ml of warm 4 N aqueous potassium hydroxide. To this solution were added 50 ml of isopropyl alcohol and 45 ml of isopropyl iodide, the biphasic system emulsified and held in this condition for 12 hours at 55°. Agitation was discontinued, and the aqueous phase was collected and treated as described for the preparation of the *O*-ethyl analog. The crude product was recrystallized from hot water and then from 50% aqueous methanol to give 8.3 g (52%) of α-*N*-acetyl-*O*-isopropyl-L-tyrosine, fine colorless needles, mp 184.0–185.0°, $[\alpha]_D^{25}$ 55.5 ± 2.5° (*c*, 0.35% in methanol). The Folin-Denis test was negative.

Anal. Calcd. for $C_{14}H_{19}O_4N$ (265): C, 63.4; H, 7.2; N, 5.3. Found: C, 63.4; H, 7.3; N, 5.2.

The preceding product was also prepared via a route involving only crystalline intermediates. L-Tyrosine, 14.5 g, was converted to the methyl ester hydrochloride (Brenner and Huber, 1953), the hydrochloride was dissolved in water, and the solution was cooled in an ice bath and exactly neutralized with 4 N aqueous sodium hydroxide. The precipitated ester was collected, washed with ice water, and dried to give 15.0 g (96%) of L-tyrosine methyl ester, mp 133.5–135.5°. The ester, 15.0 g, was dissolved in 150 ml of 10% aqueous acetic acid and the solution was filtered. To the chilled filtrate was added 12 ml of acetic anhydride, and the precipitated product was collected, washed with ice water, dried *in vacuo*, and then dissolved in methanol. The solution was decolorized with Norit and filtered, and the filtrate was evaporated *in vacuo*. The syrupy residue crystallized to give 14.0 g (77%) of α-*N*-acetyl-L-tyrosine methyl ester, mp 134–136°. The monohydrate has a mp of 118–120° (Jackson, 1952). Alkylation of 14.0 g of α-*N*-acetyl-L-tyrosine methyl ester with 66 ml of 4 N aqueous sodium hydroxide, 66 ml of isopropyl alcohol, and 22 ml of isopropyl iodide essentially as described above led to a crude product which was recrystallized twice from 50% aqueous methanol to give 11.8 g (75%) of α-*N*-acetyl-*O*-isopropyl-L-tyrosine, fine needles, mp 184.0–185.0°, $[\alpha]_D^{24}$ 54.7° (*c*, 0.5% in methanol).

α-N-Acetyl-O-isopropyl-L-tyrosine Methyl Ester.—Esterification of 39 g of α-*N*-acetyl-*O*-isopropyl-L-tyrosine with methanol and thionyl chloride (Brenner and Huber, 1953), followed by evaporation of the excess methanol *in vacuo*, led to a syrupy product. The syrup was dissolved in ethyl acetate, and the ethyl acetate solution was washed with dilute aqueous sodium carbonate, dilute aqueous hydrochloric acid, and water, and dried over Drierite. The solution was evaporated *in vacuo* to give a yellow viscous oil, the oil was dissolved in a mixture of ethyl ether and 30–60° ligroin, the solution was decolorized with Norit and filtered, and the filtrate was freed of solvent by evaporation *in vacuo* at 25° or below to give 30.5 g (74%) of crude ester, mp 58–59°, $[\alpha]_D^{25}$ 32.4° (*c*, 1.1% in methanol). The crude product was dissolved in a small volume of ethyl ether, the clear solution was cooled in an ice-salt bath, and sufficient 30–60° ligroin was added to facilitate crystallization of α-*N*-acetyl-*O*-isopropyl-L-tyrosine methyl ester, clusters of fine needles, mp 59.0–60.0°, $[\alpha]_D^{25}$ 34.0 ± 0.4° (*c*, 1.9% in methanol).

Anal. Calcd. for $C_{15}H_{21}O_4N$ (284): C, 64.5; H, 7.6; N, 5.0. Found: C, 64.6; H, 7.7; N, 5.0.

Kinetic Studies.—All kinetic studies were performed with the aid of a *p*H-stat as described previously (Applewhite *et al.*, 1958a,b). The standard reaction system consisted of 10.0 ml of solution: 1.0 ml of enzyme stock solution added to 1.0 ml of 1.0 M aqueous sodium chloride and 1.0–8.0 ml of substrate stock solution with water added where appropriate. Solutions were prepared with preboiled, CO_2-free distilled water, and a stream of nitrogen was passed over the reaction vessel during titration. The dP/dt recorder traces were corrected for the nonenzyme-catalyzed hydrolysis of substrate. No correction was made for an enzyme blank reaction because of the low values of K_0 obtained for all substrates. All computations were performed on a Datatron 220 digital computer programmed as described previously (Abrash, *et al.*, 1960). The α-chymotrypsin used, bovine, salt-free, Armour lot T-97209, was analyzed by a micro Kjeldahl procedure and found to contain 14.6% nitrogen on an as-is basis. Other pertinent experimental details are summarized in Table I.

RESULTS

The α-chymotrypsin-catalyzed hydrolysis of α-*N*-methyl and of *O*-methyl, ethyl, and isopropyl α-*N*-acetyl-L-tyrosine methyl ester, in aqueous solutions at 25.0°, *p*H 7.90, and 0.10 M in sodium chloride is described by equation (1).

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

The values obtained for the constants k_0 and K_0 of equation (1) for each of the four substrates are given in Table I.

DISCUSSION

Before proceeding with a discussion of the data summarized in Table I it is necessary to establish a

point of reference. The obvious reference compound is α-*N*-acetyl-L-tyrosine methyl ester. However, this compound is so reactive, under the conditions used for evaluation of its α-*N*- and *O*-alkyl derivatives, that we have been unable to evaluate its kinetic constants with presently available methods. Fortunately, it is possible to arrive at a reasonable estimate of the magnitudes of the constants k_0 and K_0 for this substrate. It is known that for the α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetyl-L-phenylalanine methyl ester, under the conditions employed in the present study, $k_0 = 52.5 \pm 15.8\ \text{sec}^{-1}$, $K_0 = 1.25 \pm 0.95$ mM and $k_0/K_0 = 42{,}000\ \text{M}^{-1}\ \text{sec}^{-1}$ (Jones and Niemann, 1963). From the data summarized by Neurath and Hartley (1959) we expect α-*N*-acetyl-L-tyrosine methyl ester to be a more reactive substrate than α-*N*-acetyl-L-phenylalanine methyl ester and are thus led to assume values of $k_0 = 100\ \text{sec}^{-1}$, $K_0 = 1\ \text{m}M$ and $k_0/K_0 = 100{,}000\ \text{M}^{-1}\text{sec}^{-1}$ for the former compound. One can be confident that these values are accurate as to orders of magnitude.

Neurath and Schwert (1950), drawing upon earlier observations of Kaufman and Neurath (1949a,b), noted that α-*N*-phthaloyl-DL-phenylalanine methyl ester was incapable of functioning as a substrate of α-chymotrypsin. From this and other evidence Neurath and Schwert (1950) were led to the view that the α-amido hydrogen atom in an α-*N*-acylated α-amino acid derivative participated in hydrogen bonding of substrate to the enzyme. However, it should be noted that the observations of Seligman and Wolf (1951) led these investigators to characterize α-*N*-phthaloyl-DL-phenylalanine β-naphthol ester as a substrate of moderate reactivity.

Kuk-Meri and Lichtenstein (1957) reported that α-*N*-methyl-D- and L-phenylalanine ethyl ester were not hydrolyzed by α-chymotrypsin and Wolf and Niemann (1963b) noted that, in contrast to acetylglycine methyl ester, acetyl-*N*-methylglycine methyl ester was incapable of functioning as a substrate of this enzyme. However, interpretation of the latter observation in terms of abolition of hydrogen bonding involving the α-amido hydrogen atom lost much of its force when it was found that the kinetic constants for the α-chymotrypsin-catalyzed hydrolysis of acetylglycine methyl ester and of methyl levulinate were essentially indistinguishable (Wolf and Niemann, 1963b).

The ambiguity inherent in the above results led us to consider a system where the consequences of replacement of the α-amido hydrogen atom by a methyl group could be more clearly delineated. We were thus led to a comparison of the kinetic properties of α-*N*-acetyl-L-tyrosine methyl ester and α-*N*-acetyl-α-*N*-methyl-L-tyrosine methyl ester.

Recalling our previous estimate of $K_0 = 1$ mM, $k_0 = 100\ \text{sec}^{-1}$ and $k_0/K_0 = 100{,}000\ \text{M}^{-1}\ \text{sec}^{-1}$ for the kinetic constants of α-*N*-acetyl-L-tyrosine methyl ester, it is evident from the value of k_0/K_0 given in Table I that replacement of the α-amido hydrogen atom by a methyl group results in a loss in reactivity of the order of 10^4. It is also evident that this loss in reactivity is largely a consequence of a marked diminution in the value of k_0 since the increase in K_0 is but an order of magnitude or less.

Before proceeding with our interpretation of the above results it is necessary to inquire into the physical significance of the constant K_0 of equation (1). Although there is abundant evidence supporting the view that for the so-called specific substrates of α-chymotrypsin this constant approximates the enzyme-substrate dissociation constant (Neurath and Hartley, 1959), Zerner and Bender (1963) in a recent communication take the position that while this is true for α-*N*-acetyl-L-tryptophanamide it is not so for corresponding esters. Specifically for the representation,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{\displaystyle P_1}{\overset{}{ES' + }} \xrightarrow{k_3} E + P_2, \qquad (2)$$

where $K_m = k_{-1}/k_1$, Zerner and Bender (1963) asssume that for α-*N*-acetyl-L-tryptophanamide $K_0 = K_m$ and $k_0 = k_2$, but for α-*N*-acetyl-L-tryptophan methyl, ethyl, and *p*-nitrophenyl esters $K_0 = K_m/(1 + k_2/k_3)$ and $k_0 = k_2/(1 + k_2/k_3)$, i.e., $K_0 = K_m/(k_2/k_0)$ with k_3 assumed to be the rate-controlling step. When $k_3 \doteq k_2$, $k_{-1} > k_2$, and $k_1 \geq k_3$ an increase in $k_0 = [k_2k_3/(k_2 + k_3)]$ will cause $K_0 = [(k_{-1} + k_2)/k_1][k_3/(k_2 + k_3)]$ to decrease (Hein and Neimann, 1961). However, when the preceding conditions are not satisfied it no longer follows that K_0 must decrease when k_0 increases. Thus, the argument used by Zerner and Bender (1963) to arrive at the conclusion that $K_0 \neq K_m$ for the esters may be questioned. Furthermore, it will be seen from the data given in Table I that with the *O*-alkyl derivatives of α-*N*-acetyl-L-tyrosine methyl ester order of magnitude increases in values of k_0 are associated with relatively *minor increases* in those of K_0. Finally, if one compares the constants for α-*N*-acetyl-*O*-isopropyl-L-tyrosine methyl ester with those estimated for α-*N*-acetyl-L-tyrosine methyl ester, one finds that a 10^4 decrease in the value of k_0 has no significant effect upon that of K_0. We are thus led to the conclusion that at least for the α-*N*-acylated-α-amino acid esters considered in this communication there is no compelling evidence to regard K_0 as other than an apparent enzyme-substrate dissociation constant, i.e., $K_0 \doteq K_m$.

Returning to the interpretation of the kinetic behavior of α-*N*-acetyl-α-*N*-methyl-L-tyrosine methyl ester we may now conclude that replacement of the α-acetamido hydrogen atom of α-*N*-acetyl-L-tyrosine methyl ester by a methyl group has relatively little effect on the ability of the modified molecule to bind to the active site of the enzyme. This behavior is in accord with that expected from structural considerations (Hein and Niemann, 1961, 1962). Both molecules are of the type ($S^{3E}_{R_2R_3}$) in which the constant K_0 is principally determined by interaction of the carbomethoxy (COR_3) and *p*-hydroxybenzyl (R_2) groups with their respective complementary loci (ρ_3 and ρ_2) at the active site.

Hein and Niemann (1961, 1962) have noted that with substrates of the $S^{3E}_{R_2R_3}$ limit type the α-acylamino group (R_1) functions primarily in an orienting sense, an observation which led to the postulate: "An acylamino group is required in the ρ_1 position for high values of k_0. Its removal or replacement by less polar or less constrained groups can decrease values of k_0 by factors up to 10^4." The greater than 10^3-fold decrease in the value of k_0 associated with replacement of an α-acetamido by an α-*N*-methylacetamido group provides additional support for the conclusions of Hein and Niemann (1961, 1962). While consistent with the proposition that interaction of the α-acylamino group with its complimentary locus at the active site involves hydrogen bond formation in which the amido group functions as a proton donor (Neurath and Schwert, 1950) our observations afford no proof of the validity of this assertion. The observed diminution in the value of k_0 could arise from a steric effect.

Steric effects arising from the structure of the substrate molecule have been encountered earlier

(Almond *et al.*, 1962; Wolf and Niemann, 1963b; Waite and Niemann, 1962; Jones and Niemann, 1962; Abrash, 1961). They have arisen from replacement of the α-hydrogen atom by a bulkier group or by β-branching of the side chain of both aliphatic and aromatic α-*N*-acylated-α-amino acid derivatives. It is evident from the data given in Table I for the three *O*-alkyl derivatives of α-*N*-acetyl-L-tyrosine methyl ester that a new and major steric effect has been discovered.

The nature of this effect may be inferred from the constants k_0 and K_0. Relative to α-*N*-acetyl-L-tyrosine methyl ester replacement of its phenolic hydrogen atom by an ethyl, methyl, or isopropyl group leads to an increase in values of K_0 of an order of magnitude or less, the effect being greatest for an ethyl and least for an isopropyl group. Thus this replacement has relatively little effect on the ability of the altered molecule to combine with the active site of the enzyme. However, in the series *O*-ethyl, -methyl, and -isopropyl, values of k_0 are diminished in steps of an order of magnitude. The dramatic diminution in values of k_0 can be appreciated when it is realized that the value for α-*N*-acetyl-*O*-isopropyl-L-tyrosine methyl ester is of the same order of magnitude as that of acetylglycine methyl ester.[1] Here an α-*N*-acylated aromatic α-amino acid methyl ester, with a side chain devoid of β-branching and containing an α-hydrogen atom, yields an enzyme-substrate complex that is converted into products at a rate comparable to one arising from an analogous substrate having no side chain. We conclude that while α-*N*-acetyl-*O*-isopropyl-L-tyrosine methyl ester can effectively combine with the active site of the enzyme, it generally does so in an orientation in which nucleo- and/or electrophilic attack on the carbonyl group of the hydrolyzable carbomethoxy component is sterically unfavorable.

At this time we are unable to explain the unusual order observed for values of both K_0 and k_0, i.e., ethyl > methyl > isopropyl, and to specify in greater detail the nature of the binding modes of this class of substrates. However, it is clear that the earlier suggestion (Neurath and Schwert, 1950) that "introduction of a negative substituent into the aromatic ring causes an increase in susceptibility to hydrolysis" of α-*N*-acylated aromatic α-amino acid derivatives is valid only when steric factors do not intervene. One such factor has been disclosed in this communication.

[1] Wolf and Niemann (1963a) give a value of $k_0 = 0.013 \pm 0.002$ sec^{-1} in 0.5 M sodium chloride. Further studies lead to a value of $k_0 = 0.0067 \pm 0.0005$ sec^{-1} for the same system.

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