enzyme for 18 hr. and the liberated amino acids were determined. The results are shown in Table IX. The above results established C-4 to be val·try [*ser·ser·phe·glu·ser·ser·ser·gly·leu·val·try·thr* (*ser, gly, pro, ala*) *thr*].

From the work of Niu and Fraenkel–Conrat[21] already cited, the sequence of peptide C-5 could be assumed to be thr·ser·gly·pro·ala·thr. This sequence was confirmed. About 0.2 μmole of peptide C-5 in 0.2 ml. of buffer was treated with 0.35 C_l unit of leucine aminopeptidase for 20 hr. and the liberated amino acids were determined. The N-terminal threonine and one mole of serine were released. A partial sequence for peptide C-5 of thr·ser (gly, pro, ala) thr could thus be written. About 10 μmoles of C-5 in 10 ml. of buffer was treated with about 0.7 C_l unit of leucine aminopeptidase for 24 hr. About 0.35 C_l unit of the enzyme was added and the incubation was continued for an additional 24 hr. This treatment completely removed the N-terminal threonine and the adjacent serine. The residual peptide was purified first by paper chromatography on Whatman 3 MM paper and then by electrophoresis on the same type paper with 33⅓% acetic acid as buffer. The amino acid composition of the purified peptide was gly, 0.93, pro, 1.00, ala, 1.08, thr, 1.00. This peptide then was degraded two steps by the Edman technique and the amino acid composition of the residual peptide after each step was determined. Found: Step 1, gly, 0.34, pro, 0.89, ala, 1.00, thr, 1.05; step 2, gly, 0.20, pro, 0.18, ala, 1.00, and thr, 0.97. Although the cleavage at step 1 was only about 70% complete, these results conclusively showed that the sequence of the peptide was gly·pro·ala·thr (threonine was known to be C-terminal from the treatment of Peptide 12 with carboxypeptidase A). The sequence of peptide C-5 thus was established to be thr·ser·gly·pro·ala·thr, confirming the sequence postulated by Niu and Fraenkel-Conrat. The sequence of Peptide 12 therefore was established to be ser·ser·phe·glu·ser·ser·ser·gly·leu·val·try·thr·ser·gly·pro·ala·thr.

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[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA]

Rearrangement of α-N-Acetyl-L-tyrosinhydrazide to 1-Acetyl-2-(L-tyrosyl)-hydrazine[1]

BY ABRAHAM N. KURTZ AND CARL NIEMANN[2]

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When an aqueous hydrochloric acid solution of α-N-acetyl-L-tyrosinhydrazide is heated, the preceding compound is rearranged, in good yield, to 1-acetyl-2-(L-tyrosyl)-hydrazine. Both the reaction and its product are of interest. The reaction offers promise of providing a new means for the identification of the carboxyl terminal α-amino acid residues of peptides and possibly for the stepwise degradation of peptides from their carboxyl terminal end. The product is the most effective readily reversible inhibitor of α-chymotrypsin discovered to date.

Aqueous solutions of α-N-acetyl-L-tyrosinhydrazide, a relatively water insoluble substrate of α-chymotrypsin,[3,4] are stable at 95–100° for periods up to and possibly exceeding 3 hr. However, when aqueous acidic solutions of the substrate were similarly treated a new and very water soluble species was formed. This latter substance was not a substrate but an inhibitor with an apparent enzyme–inhibitor dissociation constant at *p*H 7.9 smaller than any recorded previously.[5] It will be recalled that at *p*H 7.9 L-tyrosinhydrazide is a substrate of α-chymotrypsin,[4] that α-N-acetyl-L-tyrosine is a relatively poor inhibitor and that L-tyrosine is relatively insoluble in dilute aqueous acid.

An acidic solution of α-N-acetyl-L-tyrosinhydrazide maintained at 95–100° exhibited two parallel phenomena. One, the degree of inhibition of the α-chymotrypsin catalyzed hydrolysis of methyl acetyl-L-valinate in aqueous solutions at 25° and *p*H 7.90 and 0.1 *M* in sodium chloride by aliquots of the solution increased regularly with increased time of heating. Two, potentiometric titration, at 25°, of aliquots of the solution, showed that the concentration of the original conjugate acid, of $pK_A' = 3.2$, decreased while that of a new conjugate acid, of $pK_A' = 7.0$, increased with increased time of heating.

The kinetics of the above transformation were followed both enzymatically and potentiometrically. An aqueous solution 0.05 *M* in α-N-acetyl-L-tyrosinhydrazide and 0.05 *M* in hydrochloric acid was maintained at 96 ± 1°. Aliquots of the reaction mixture were removed at various time intervals and titrated potentiometrically at 25° with aqueous 0.125 *N* sodium hydroxide with the aid of a Di-Functional Recording Titrator.[6] The titer due to the appearance of the less acidic conjugate acid was calculated from the titration curves. Similarly, aliquots were assayed for inhibitor content using the system α-chymotrypsin–methyl acetyl-L-valinate, in aqueous solutions at 25.0° and *p*H 7.90 and 0.10 *M* in sodium chloride, with $[S]_0 = 40 \times 10^{-3}$ *M* and [E] = 0.1464 mg. protein-nitrogen per ml. Totally competitive inhibition was assumed and the size of the aliquot was adjusted to correspond to a maximum concentration of $[I] = 1.0 \times 10^{-3}$ *M* in the enzyme–substrate–inhibitor system, *i.e.*, for the initial reaction system at $t = 0$ the concentration of α-N-acetyl-L-tyrosinhydrazide was 1×10^{-3} *M* and at $t = \infty$, assuming 1:1 stoichiometry for the formation of the inhibitor, the concentration of the inhibitor was

(1) Supported in part by a grant from the National Institutes of Health, U. S. Public Health Service.

(2) To whom inquiries regarding this article should be sent.

(3) R. V. MacAllister and C. Niemann, *J. Am. Chem. Soc.*, **71**, 3854 (1949).

(4) R. Lutwack, H. F. Mower and C. Niemann, *ibid.*, **79**, 2179, 5690 (1957).

(5) R. J. Foster, H. J. Shine and C. Niemann, *ibid.*, **77**, 2378 (1955).

(6) The instrument used was manufactured by the International Instrument Co., Canyon, Calif. It can be used for constant or variable *p*H titrations.

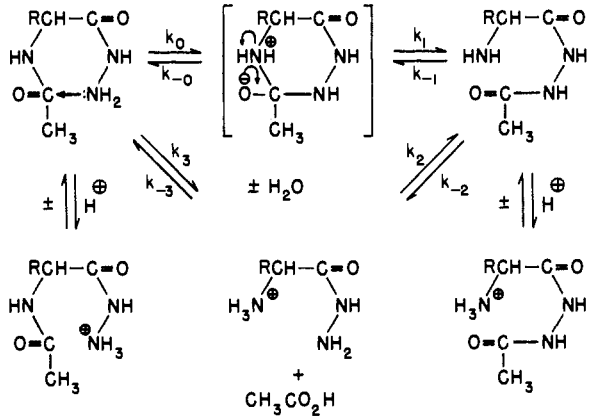

Fig. 1.—Rearrangement of α-N-acetyl-L-tyrosinhydrazide to 1-acetyl-2-(L-tyrosyl)-hydrazine: R is $-CH_2C_6H_4OH$-*p*.

1×10^{-3} *M*. This precaution was necessary because α-N-acetyl-L-tyrosinhydrazide is a substrate and can compete with methyl acetyl-L-valinate for the enzyme. It is fortunate that the value of K_S for α-N-acetyl-L-tyrosinhydrazide is $29.5 \pm 6.0 \times 10^{-3}$ *M*[7] and that of K_I for the inhibitor derived from it was estimated to be 9×10^{-5} *M*. With this difference in the magnitudes of the two constants, a maximum concentration of α-N-acetyl-L-tyrosinhydrazide of 1×10^{-3} *M* and an initial concentration of methyl acetyl-L-valinate of 40×10^{-3} *M*, the contribution of α-N-acetyl-L-tyrosinhydrazide to the over-all behavior of the enzyme system can be ignored.

In the region of 0 to 80% conversion the rate of appearance of conjugate acid of $pK_A' = 7.0$ was first order with respect to the concentration of α-N-acetyl-L-tyrosinhydrazide and the rate constant $k = 0.028$ min.$^{-1}$. In the region of 0–65% conversion the rate of appearance of inhibitor of $K_I = 9 \times 10^{-5}$ *M* was also first order with respect to the concentration of α-N-acetyl-L-tyrosinhydrazide with $k = 0.025$ min.$^{-1}$. When the extent of conversion exceeded 65% the rate of inhibitor formation decreased, when evaluated by enzymatic assay, whereas the rate of appearance of conjugate acid of $pK_A' = 7.0$ remained reasonably first order. The order of the reaction with respect to the concentration of hydrogen ion was not determined.

At this point it was assumed that the conjugate acid of $pK_A' = 7.0$ and the inhibitor of $K_I = 9 \times 10^{-5}$ *M* was 1-acetyl-2-(L-tyrosyl)-hydrazine and that it was formed from α-N-acetyl-L-tyrosinhydrazide by an N- to N′-acyl migration.

The reason for the diminution in the rate of formation of inhibitor after 65% conversion became apparent when it was found that a reaction mixture carried beyond 65% conversion contained L-tyrosinhydrazide. This latter product could arise by deacetylation of α-N-acetyl-L-tyrosinhydrazide or of 1-acetyl-2-(L-tyrosyl)-hydrazine, either separately or simultaneously.

As expected[8] the value of pK_A' for the first conjugate acid of L-tyrosinhydrazide was found to be 7.1, which is very close to that of 1-acetyl-2-(L-tyrosyl)-hydrazine. Thus, if the former product arose from a secondary reaction involving deacetylation of the latter, the reaction would remain first order with respect to the rate of appearance of conjugate acids of $pK_A' = 7.0 \pm 0.1$ but would not continue to be first order with respect to the rate of formation of inhibitor $K_I = 9 \times 10^{-5}$ *M* because the K_S value of L-tyrosinhydrazide is known to be $6 \pm 4 \times 10^{-3}$ *M*.[4] Alternatively, if a secondary reaction involving deacetylation of α-N-acetyl-L-tyrosinhydrazide were involved, one would still obtain the same result. Depletion of the concentration of α-N-acetyl-L-tyrosinhydrazide *via* the secondary reaction would be without effect upon the rate of formation of conjugate acids of $pK_A' = 7.0 \pm 0.1$ but would cause a diminution in the rate of appearance of inhibitor of $K_I = 9 \times 10^{-5}$ *M* because this latter reaction would be competitive with that involving deacetylation of α-N-acetyl-L-tyrosinhydrazide to L-tyrosinhydrazide.

The presence of L-tyrosinhydrazide in the reaction mixture raised the question as to whether 1-acetyl-2-(L-tyrosyl)-hydrazine could be formed from the former compound and acetic acid.

An aqueous solution 0.05 *M* in L-tyrosinhydrazide, hydrochloric acid and acetic acid was maintained at 95 ± 1° for 4 hr. Enzymatic assay indicated that not more than 6% of inhibitor of $K_I = 9 \times 10^{-5}$ *M* had been formed. Since reaction mixtures initially 0.05 *M* in α-N-acetyl-L-tyrosinhydrazide and hydrochloric acid and maintained at 95 ± 1° for 3 hr. were 0.040 to 0.045 *M* in 1-acetyl-2-(L-tyrosyl)-hydrazine, based upon enzymatic assay, it may be inferred that the latter compound is formed from the former by an intramolecular reaction rather than by an improbable intermolecular reaction involving deacetylation of α-N-acetyl-L-tyrosinhydrazide to L-tyrosinhydrazide followed by acetylation of the latter compound to give 1-acetyl-2-(L-tyrosyl)-hydrazine.

The preceding observations are summarized in Fig. 1, where R is $-CH_2C_6H_4$-OH-*p*. In this scheme are represented a series of equilibria displaced toward protonated species of $pK_A' = 7.0 \pm 0.1$. α-N-Acetyl-L-tyrosinhydrazide is presumed to be transformed into a cyclic intermediate, which may or may not lose a molecule of water to form a 3,5-disubstituted 1,2,4-triazolone-6,[9,10] and this intermediate, or its dehydration product, then converted into 1-acetyl-2-(L-tyrosyl)-hydrazine. Insofar as the over-all reaction is concerned it may be recalled that DL-α-phthalamamido-β-phenylthiopropionamide is transformed readily into phthalimide and the hydrochloride of DL-phenylalaninthioamide by reaction with boiling methanolic hydrogen chloride.[11] Bender[12] reports a value of $k = 0.014$ min.$^{-1}$ for the intramolecular hydrolysis of phthalamic acid at 47.3° and extrapolation to 95°[13] gives a value of $k = 1.1$ min.$^{-1}$.

(7) J. T. Braunholtz, R. J. Kerr and C. Niemann, *J. Am. Chem. Soc.*, **81**, 2852 (1959).

(8) H. R. Almond, Jr., R. J. Kerr and C. Niemann, *ibid.*, **81**, 285 (1959).

(9) O. Widman, *Ber.*, **26**, 2612 (1893).

(10) M. Sen, *J. Ind. Chem. Soc.*, **6**, 1001 (1929).

(11) P. E. Peterson and C. Niemann, *J. Am. Chem. Soc.*, **79**, 1389 (1957).

(12) M. L. Bender, *ibid.*, **80**, 5380 (1958).

(13) With $\Delta H^* = 20.7$ kcal./mole and $\Delta S^* = -12.1$ e.u.[12]

While this value is *ca.* forty times greater than the value of k = 0.025–0.028 min.$^{-1}$ observed for the case at hand, the magnitude of the latter value is not unreasonable for an intramolecular reaction of the kind envisioned.

The pK_A' value of the conjugate acid of α-N-acetyl-L-tyrosinhydrazide was found to be 3.2. Thus, under the reaction conditions employed, the concentration of the corresponding base, *i.e.*, α-N-acetyl-L-tyrosinhydrazide, is approximately one-fourth of that of its conjugate acid. This fact may in part be responsible for the lesser magnitude of the rate constant observed in this study if, as it seems reasonable, the formation of 1-acetyl-2-(L-tyrosyl)-hydrazine proceeds by intramolecular nucleophilic attack of the acetyl carbonyl carbon atom by the terminal nitrogen atom of the hydrazide with a concomitant migration of a proton to the α-nitrogen atom, as indicated in Fig. 1. Since the pK_A' of the conjugate acid of 1-acetyl-2-(L-tyrosyl)-hydrazine was found to be 6.93, *vide ante*, it follows that the over-all favorable energy of the system is derived from the large enthalpy change of *ca.* 10–12 kcal./mole[14] accompanying protonation of the α-amino group of 1-acetyl-2-(L-tyrosyl)-hydrazine.

An aqueous solution 0.05 M in α-N-acetyl-L-tyrosinhydrazide and hydrochloric acid was heated for 3 hr. at 95 ± 1°, an excess of β-naphthalenesulfonic acid added and the reaction mixture heated for an additional hour to effect solution of the sulfonic acid. The product isolated in 39% yield proved to be the relatively insoluble L-tyrosinhydrazide di-β-naphthalene sulfonate salt despite the fact that enzymatic assay of the reaction mixture prior to the addition of the sulfonic acid indicated that α-N-acetyl-L-tyrosinhydrazide had been converted into 1-acetyl-2-(L-tyrosyl)-hydrazine in 85% yield.

Potentiometric titration of the di-β-naphthalene sulfonate salt gave an equivalent weight of 630 ± 2 and values of pK_{A1}' = 3.2 ± 0.02 and pK_{A2}' = 7.12 ± 0.02. The expected range for pK_A' values of an α-ammonium group present in the conjugate acid of an α-amino acid hydrazide is 7.4 ± 0.4.[8] Hence the value of pK_{A1}' = 3.2 ± 0.02 may be assigned to the terminal hydrazide ammonium group and the value of pK_{A2}' = 7.12 ± 0.02 to the α-ammonium group of the conjugate diacid of L-tyrosinhydrazide.

The isolation of L-tyrosinhydrazide di-β-naphthalene sulfonate salt in 39% yield from a reaction mixture initially containing 85% of 1-acetyl-2-(L-tyrosyl)-hydrazine is due to the relative insolubility of the former compound. When an aqueous solution 0.05 M in α-N-acetyl-L-tyrosinhydrazide and β-naphthalene sulfonic acid was heated at 95 ± 1° for 3 hr., the water-soluble 1-acetyl-2-(L-tyrosyl)-hydrazine β-naphthalene sulfonate salt was isolated in 45% yield. Although soluble in water and ethanol, the mononaphthalene sulfonate salt can be recrystallized from propanol-2. Potentiometric titration gave an equivalent weight of 440 ± 4 and a value of pK_A' = 6.93. These experiments demonstrate that the pathways leading to deacetylation of 1-acetyl-2-(L-tyrosyl)-hydrazine and precipitation of the L-tyrosinhydrazide di-β-naphthalene sulfonate salt can be blocked if the solubility product of the latter salt is not exceeded during the acyl migration or subsequent isolation procedures.

After much effort 1-acetyl-2-(L-tyrosyl)-hydrazine was obtained as the free base. It was found to be quite soluble in water, methanol and ethanol and could be recrystallized from propanol-2. It was relatively insoluble in all of the common organic solvents except the lower molecular weight alcohols. Aqueous solutions of the free base gave a positive ninhydrin reaction (red color) and failed to react with benzaldehyde. As with the crude reaction mixture discussed previously, attempts to precipitate a mono-β-naphthalene sulfonate salt resulted in deacetylation and the formation of L-tyrosinhydrazide di-β-naphthalene sulfonate salt.

Acetylation of 1-acetyl-2-(L-tyrosyl)-hydrazine with acetic anhydride and aqueous sodium hydroxide at 0° gave 1-acetyl-2-(O,N-diacetyl-L-tyrosyl)-hydrazine in 90% yield. This product was identical with that obtained by a comparable acetylation of α-N-acetyl-L-tyrosinhydrazide and upon partial hydrolysis gave 1-acetyl-2-(α-N-acetyl-L-tyrosyl)-hydrazine.

Potentiometric titration of an aqueous solution of 1-acetyl-2-(L-tyrosyl)-hydrazine gave a value of pK_A' = 6.93 ± 0.02 and when evaluated as a competitive inhibitor of the α-chymotrypsin catalyzed hydrolysis of methyl acetyl-L-valinate, proceeding as before, a value of K_I = 7.4 ± 0.3 × 10^{-5} M was obtained. These values are in good agreement with those derived from the crude reaction mixture, *i.e.*, pK_A' = 7.0 and K_I = 9 × 10^{-5} M, and indicate that under the reaction conditions employed α-N-acetyl-L-tyrosinhydrazide was transformed into 1-acetyl-2-(L-tyrosyl)-hydrazine in yields of 80–85%.

The ultraviolet spectrum of 1-acetyl-2-(L-tyrosyl)-hydrazine was compared with that of α-N-acetyl-L-tyrosinhydrazide. In 0.02 M aqueous hydrochloric acid the spectra were identical with maxima at 224 mμ (ε 9500), 276 mμ (ε 1450) and 281 mμ (ε 1250). In 0.01 M aqueous sodium hydroxide both compounds exhibited maxima at 240 and 294 mμ. However, with α-N-acetyl-L-tyrosinydrazide ε = 25,000 at 240 mμ and 5600 at 294 mμ whereas with 1-acetyl-2-(L-tyrosyl)-hydrazine ε = 21,000 at 240 mμ and 2500 at 294 mμ. The lesser molar extinction coefficient in the latter instance suggests that the phenolic hydroxyl group in 1-acetyl-2-(L-tyrosyl)-hydrazine is less acidic than that in α-N-acetyl-L-tyrosinhydrazide.

The infrared spectra of the above compounds were determined in Nujol mulls. The original hydrazide gave maxima at 3268, 1664, 1613, 1513, 1299, 1271 and 1242 cm.$^{-1}$ and the rearranged compound maxima at 3333(sh.), 3215(s.), 1664(sh.w.), 1616, 1585, 1515, 1299(w.) and 1258 cm.$^{-1}$. In addition, the latter compound in a hexachlorobutadiene-1,3 mull gave a peak at 1481 cm.$^{-1}$ which was not observable in a Nujol mull.

(14) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides as Ions and Dipolar Ions," Reinhold Publ. Corp., New York, N. Y., 1943.

The infrared spectra of α-N-acetyl-L-tyrosinhydrazide differs from that of 1-acetyl-2-(L-tyrosyl)-hydrazine in several respects. One, the 1664 cm.$^{-1}$ (C=O) absorption, which is prominent in the former compound, is very weak in the latter. Two, a band at 1585 cm.$^{-1}$ is present only in the latter compound. Three, the doublet at 1271 and 1242 cm.$^{-1}$ present in the former compound, and characteristic of α-acylamino acid derivatives,[15] is replaced in the latter by a singlet at 1258 cm.$^{-1}$. Four, the shift of the N–H stretching mode from 3268 cm.$^{-1}$ in the former compound to 3215 cm.$^{-1}$ in the latter, along with its diminished carbonyl absorption, suggests that in the solid state 1-acetyl-2-(L-tyrosyl)-hydrazine assumes a cyclic *cis* structure. In both compounds, the presence of NH_2NH deformation frequencies at 1613–1616 cm.$^{-1}$ is evident.

The preceding observations demonstrate that the rearrangement product of α-N-acetyl-L-tyrosinhydrazide is 1-acetyl-2-(L-tyrosyl)-hydrazine. Of the possible products arising from the reaction of α-N-acetyl-L-tyrosinhydrazide with aqueous acid, those containing an α-amino group can be distinguished readily by means of the ninhydrin reaction. In this reaction 1-acetyl-2-(L-tyrosyl)-hydrazine gives a red color, L-tyrosinhydrazide a purple color and L-tyrosine an azure blue color. α-N-Acetyl-L-tyrosinhydrazide, the starting material gives a pale yellow color.

Aqueous solutions of 1-acetyl-2-(L-tyrosylhydrazine (*p*H *ca.* 8) are stable at 95–100° for periods up to and possibly exceeding 4 hr. Thus, under these conditions rearrangement of 1-acetyl-2-(L-tyrosyl)-hydrazine to α-N-acetyl-L-tyrosinhydrazide, or hydrolytic cleavage of the former compound to L-tyrosinhydrazide, does not occur to any measurable extent. However, aqueous solutions of α-N-acetyl-L-tyrosinhydrazide buffered at *p*H 8 give upon gentle warming a product that reacts with ninhydrin to produce the red color characteristic of 1-acetyl-2-(L-tyrosyl)-hydrazine. It therefore appears that acyl migration from α-amino nitrogen to the terminal nitrogen atom of the hydrazide function may proceed under either acidic or basic conditions. In the presence of acid the driving force of the reaction appears to be the protonation of the more basic α-amino group. In the presence of base the driving force may be provided by a greater solvent stabilization of the same group, a view that is consistent with the observation that the reverse migration cannot be observed at *p*H 8.

Preliminary experiments conducted with α-N-trimethylacetyl-L-tyrosinhydrazide and α-N-acetylglycylhydrazide have shown that rearrangement proceeds under acidic conditions in a manner analogous to that observed for α-N-acetyl-L-tyrosinhydrazide. The fact that the rate of rearrangement of the first compound is much slower and that of the second is comparable to that observed for α-N-acetyl-L-tyrosinhydrazide, offers further evidence in support of the proposed mechanism of intramolecular nucleophilic attack on the α-acylamino carbonyl group by the terminal hydrazide nitrogen atom.

(15) L. J. Bellamy, "The Infrared Spectra of Complex Molecules," John Wiley and Sons, Inc., New York, N. Y., 1958.

The rearrangement of α-N-acetyl-L-tyrosinhydrazide, under acidic or basic conditions, to 1-acetyl-2-(L-tyrosyl)-hydrazine is of general interest.

The azide method of peptide synthesis ordinarily requires an α-acylamino acid or peptide hydrazide as an intermediate. With the demonstration that an α-acylamino acid hydrazide may undergo acyl migration under acidic or basic conditions, it is evident that the possibility that this latter reaction may have occurred must be considered whenever hydrazides are employed as intermediates.

Hydrazinolysis of proteins has been employed more or less successfully for the identification of carboxyl terminal α-amino acid residues.[16] It is now evident that this technique, which is based upon isolation of the carboxyl terminal α-amino acid residues as the α-amino acids, all other residues presumably having been converted to the corresponding α-amino acid hydrazides, is subject to perturbation by the possibility of rearrangement of intermediate peptide hydrazides. In the absence of information relative to the hydrazinolysis of peptides of known structure, it is not possible to assess the consequences of this possible side reaction. However, it is certain that partial hydrazinolysis followed by partial acid hydrolysis will result in substantial acyl migration and the appearance of an initial non-terminal α-amino acid residue as an apparent amino terminal residue.

The acyl migration described in this communication appears to afford a new procedure for the identification of carboxyl terminal α-amino acid residues of peptides and possibly for the stepwise degradation of peptides from the carboxyl terminal end of the chain. In the first instance rearrangement of a peptide hydrazide of the general formula $H_2N(CHRCONH)_nCHR'CONHCHR''CONHNH_2$ to a diacyl hydrazine of the general formula $H_2N(CHRCONH)_nCHR'CONHNHCOCHR''NH_2$ would permit one to employ the dinitrofluorobenzene technique of Sanger[17] first on the original peptide, or its hydrazide, and then on the rearranged product to identify in turn first the amino terminal α-amino acid residue and then both the amino and carboxyl terminal α-amino acid residues. In the second instance, oxidation of the diacylhydrazine with cupric ion should lead to the original peptide deprived of its carboxyl terminal α-amino acid residue and that α-amino acid.

Finally, the observation that 1-acyl-2-(α-aminoacyl)-hydrazines may be obtained by the rearrangement of the corresponding α-acylamino acid hydrazides permits us to take advantage of the former class of compounds for the further characterization of the active site of α-chymotrypsin, and possibly other hydrolytic enzymes, through inhibition studies. As noted earlier, 1-acetyl-2-(L-tyrosyl)-hydrazine is the most effective readily reversible inhibitor of α-chymotrypsin that has been discovered to date.

Experimental

α-N-Acetyl-L-tyrosinhydrazide.—This compound was prepared by the procedure of Hogness and Niemann.[18] Lut-

(16) T. Kauffmann and F. P. Boettcher, *Ann.*, **625**, 123 (1959).

(17) F. Sanger, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 153 (1949).

wack, Mower and Niemann[4] give a m.p. of 227–228° and a value of $[\alpha]^{25}D$ 44° (*c*, 0.4% in water). The product obtained in this study had a m.p. of 232–233° (corr.) and an $[\alpha]^{25}D$ 44° (*c*, 0.36% in water).

1-Acetyl-2-(L-tyrosyl)-hydrazine.—α-N-Acetyl-L-tyrosinhydrazide, 6.0 g. (0.025 mole), was dissolved in 500 ml. of 0.05 *M* aqueous hydrochloric acid at 95°, the reaction mixture maintained at 95–98° for 3 hr. and then evaporated *in vacuo* to a syrup. The syrup was taken up in 50 ml. of methanol, the solution exactly neutralized with 1 *M* methanolic sodium methoxide, the precipitated sodium chloride removed and the solution evaporated *in vacuo* to a residue which was recrystallized from a mixture of methanol and isopropyl ether to give 2.5 g. (45%) of product, m.p. 120–140°. Two recrystallizations from acetonitrile and two from propanol-2 gave 0.71 g. (13%) of 1-acetyl-2-(L-tyrosyl)-hydrazine, hygroscopic, colorless, microscopic needles, m.p. 144–146° (corr.), $[\alpha]^{25}D$ 63 ± 2° (*c*, 1.1% in water).

Anal. Calcd. for $C_{11}H_{16}O_3N_3$ (237): C, 55.7; H, 6.4; N, 17.7. Found: C, 55.4; H, 6.4; N, 18.0.

The compound was soluble in water, methanol and ethanol but insoluble in ethyl ether, chloroform and other common organic solvents. Reaction with ninhydrin gave a red color. The compound did not react with benzaldehyde to give a hydrazone. Potentiometric titration with aqueous hydrochloric acid gave a value of $pK_A' = 6.93 \pm 0.02$.

1-Acetyl-2-(D-tyrosyl)-hydrazine.—This compound was prepared as described for the L-enantiomorph. The crude product obtained in 26% yield was thrice recrystallized from propanol-2 to give the final product, m.p. 143–144° (corr.), $[\alpha]^{25}D$ −60 ± 2 (*c* 1.1% in water).

Anal. Calcd. for $C_{11}H_{15}O_3N_3$ (237): C, 55.7; H, 6.4; N, 17.7. Found: C, 55.5; H, 6.5; N, 17.6.

1-Acetyl-2-(L-tyrosyl)-hydrazine β-Naphthalene Sulfonate Salt.—α-N-Acetyl-L-tyrosinhydrazide, 4.74 g. (0.020 mole) and 4.53 g. (0.020 mole) of β-naphthalene sulfonic acid monohydrate, that had been recrystallized from propanol-2, was dissolved in 200 ml. of water and the solution heated at 98° for 3 hr. The hot solution was decolorized with Norite, the colorless filtrate evaporated to dryness *in vacuo* and the residue recrystallized from propanol-2 to give 4.0 g. (45%) of product, m.p. 138–140°. This product was twice recrystallized from propanol-2 to give the salt, m.p. 147–149° (corr.) after preliminary softening at *ca.* 130°, $[\alpha]^{25}D$ 56 ± 2° (*c*, 2.07% in water).

Anal. Calcd. for $C_{21}H_{23}N_3O_6S$ (445): C, 56.6; H, 5.2; N, 9.4. Found: C, 56.6; H, 5.1; N, 9.4.

Potentiometric titration with aqueous sodium hydroxide gave an equivalent weight of 440, ignoring the contribution of the phenolic hydroxyl group, and a value of $pK_A' = 6.93 \pm 0.02$.

The isolation of 1-acetyl-2-(L-tyrosyl)-hydrazine as the β-naphthalene sulfonate salt as described above is a convenient procedure for obtaining a derivative of the diacylhydrazine. However, it must be noted that the β-naphthalene sulfonate anion is a competitive inhibitor of α-chymotrypsin and the use of the salt in inhibition studies introduces two inhibitors into the system.

L-Tyrosinhydrazide Di-β-naphthalene Sulfonate Salt.—Crude 1-acetyl-2-(L-tyrosyl)-hydrazine was prepared as described above. A solution of 6 g. of the crude salt contained in 100 ml. of water appeared to be 0.22 *M* with respect to 1-acetyl-2-(L-tyrosyl)-hydrazine on the basis of an enzymatic assay. To 50 ml. of this solution was added 3.0 g. (0.045 mole) of recrystallized β-naphthalene sulfonic acid monohydrate and the mixture heated on a steam-bath for 1 hr. whereupon complete solution was effected. The hot solution was decolorized with Norite, filtered and the filtrate chilled to give 2.0 g. (41%) of salt, m.p. 267–270° (corr.), $[\alpha]^{25}D$ 24 ± 1° (*c* 1.6% in dimethylformamide) after one recrystallization from water.

Potentiometric titration of the above salt with aqueous sodium hydroxide gave two end-points, the second being exactly equivalent to the first after correction for the partial ionization of the phenolic hydroxyl group. The equivalent weight was found to be 630 ± 2 (theor. 611) and $pK_{A1}' = 3.2$ and $pK_{A2}' = 7.12 \pm 0.02$. The equivalent weight found titrimetrically corresponded to a monohydrate. The salt prepared as described above was identical in every respect with that prepared directly from L-tyrosinhydrazide. Both salts when heated with ninhydrin and aqueous sodium bicarbonate gave a deep purple color.

1-Acetyl-2-(O,N-diacetyl-L-tyrosyl)-hydrazine. A. (From α-N-Acetyl-L-tyrosinhydrazide).—A solution of 4.74 g. (0.02 mole) of α-N-acetyl-L-tyrosinhydrazide in 50 ml. of water containing 0.040 mole of sodium hydroxide was stirred for 1 hr. at 0° with 4.10 g. (0.040 mole) of acetic anhydride. The precipitated product was collected, dried (yield 90%) and recrystallized from water to give the triacetyl derivative, large, colorless needles, m.p. 219–220° (corr.), $[\alpha]^{25}D$ 26 ± 1° (*c*, 0.5% in dimethylformamide). The ninhydrin test was negative.

Anal. Calcd. for $C_{15}H_{19}O_5N_3$ (321): C, 56.1; H, 6.0; N, 13.1. Found: C, 56.0; H, 6.1; N, 13.1.

B. (From 1-Acetyl-2-(L-tyrosyl)-hydrazine).—Fifty ml. of a 0.22 *M* (enzyme assay) solution of crude 1-acetyl-2-(L-tyrosyl)-hydrazine, 20 ml. of 2 *N* aqueous sodium hydroxide and 4 ml. of acetic anhydride were allowed to react at 0° as described above. The crude product again precipitated from the reaction mixture in a 90% yield and was recrystallized from water to give the triacetyl derivative, m.p. 219–222° (corr.) identical with that described immediately above; mixed m.p. 219–220° (corr.).

1-Acetyl-2-(α-N-acetyl-L-tyrosyl)-hydrazine.—1-Acetyl-2-(O,N-diacetyl-L-tyrosyl)-hydrazine, 1.35 g., was dissolved in 10 ml. of 2 *N* aqueous sodium hydroxide, the solution allowed to stand at room temperature for 1 hr. and then acidified to *p*H 3 with concd. hydrochloric acid. The reaction mixture was stored at 5° for two days, the crystalline precipitate collected and dried to give 1.05 g. of product, m.p. 228–229° (corr.). Recrystallization from aqueous propanol-2 gave 1-acetyl-2-(α-N-acetyl-L-tyrosyl)-hydrazine, m.p. 238° (corr.), $[\alpha]^{25}D$ 18 ± 1° (*c*, 1.1% in water).

Anal. Calcd. for $C_{13}H_{17}O_4N_3$ (279): C, 55.9; H, 6.1; N, 15.1. Found: C, 56.0; H, 6.1; N, 14.9.

This product depressed the melting points of α-N-acetyl-L-tyrosinhydrazide, 1-acetyl-2-(L-tyrosyl)-hydrazine and 1-acetyl-2-(O,N-diacetyl-L-tyrosyl)-hydrazine.

Methyl Acetyl-L-valinate.—The preparation has been described earlier.[19]

Kinetics of Conversion of α-N-Acetyl-L-tyrosinhydrazide to 1-Acetyl-2-(L-tyrosyl)-hydrazine.—α-N-Acetyl-L-tyrosinhydrazide, 1.20 g. (0.00506 mole), was added with stirring to 95 ml. of distilled water in a flask equipped for reflux. The temperature of the system was adjusted to 96 ± 1° and 5.0 ml. of 1.04 *M* HCl added to the solution. Five-ml. aliquots of the reaction mixture were removed at t = 8, 15, 30, 45, 60, 75, 90, 122, 150 and 180 min. and cooled in an ice-bath. One-ml. aliquots, undiluted, were titrated potentiometrically with 0.214 *N* aqueous sodium hydroxide. The titration curves showed two breaks: the first corresponded to an acid of $pK_A' = 3.2$, the second to an acid of $pK_A' = 7$. The amount of product was taken as the difference between the two end-points. First order kinetics were observed up to 80% conversion.

For the enzymatic assay, the original aliquots, taken at the times indicated, were diluted fivefold. One ml. samples of the diluted aliquots were assayed with α-chymotrypsin at [E] = 0.1464 mg. of protein-nitrogen per ml. and methyl acetyl-L-valinate at $[S]_0$ = 0.0400 *M*, NaCl = 0.100 *M*, *p*H 7.90 ± 0.01 and 25.0°. The apparent concentration of inhibitor was calculated using an assumed value of $K_I = 8.3 \times 10^{-5}$ *M*, neglecting the contributions of any other species that may have been present since K_S for the latter were >> K_I for the inhibitor. First-order kinetics were observed up to 65% conversion. The experimental data are summarized in Table I.

Hydrazine was found amongst the reaction products. It was determined spectrophotometrically as the *p*-dimethylaminobenzalazine.[4,7] For reaction times of 8, 30, 75, 122 and 180 min., the % conversion to hydrazine was 0.28, 0.52, 0.68, 0.76 and 0.82, respectively.

Attempted Reaction of L-Tyrosinhydrazide with Hydrochloric Acid and Acetic Acid.—To 100 ml. of 0.05 *M* HCl was added 0.975 g. (0.005 mole) of L-tyrosinhydrazide and 0.30 g. (0.005 mole) of glacial acetic acid. The resulting

(18) D. S. Hogness and C. Niemann, *J. Am. Chem. Soc.*, **75**, 884 (1953).

(19) T. H. Applewhite, H. Waite and C. Niemann, *ibid.*, **80**, 1465 (1958).

TABLE I

KINETICS OF THE CONVERSION OF α-N-ACETYL-L-TYROSINHYDRAZIDE TO 1-ACETYL-2-(L-TYROSYL)-HYDRAZINE AT 96°

Time (min.)	Potentiometric titration $[S]_t$[a]	ln $[S]_t$	Enzyme assay $[S]_t$[a]	ln $[S]_t$	v_0[c]
0	(5.08)	−2.98	(5.08)	−2.98	...
8	3.73	−3.29	3.85	−3.26	0.276
15	3.09	−3.48	3.13	−3.46	.191
30	2.09	−3.87	2.23	−3.80	.141
45	1.32	−4.33	1.74	−4.05	.123
60	0.89	−4.72	1.85	−3.99	.126
75	0.67	−5.01	1.60	−4.14	.119
90	..	....	1.54	−4.17	.117
105	0.34	−5.68	..	....	.116
122	.11	−6.81	1.13	−4.48	.106
150	.06	−7.42	..	....	.119
180	.00	....	..	....	.104
	k = 0.028 min.$^{-1}$ (0-98% conv).		k = 0.025 min.$^{-1}$ (0-66% conv.)		

[a] Concn. of α-N-acetyl-L-tyrosinhydrazide. Units of 10^{-2} M. [c] Units of 10^{-4} M/min.

solution in a flask equipped with reflux condenser was placed in a steam-bath (t, 95°) for 4 hr., then cooled in an ice-bath. An aliquot (0.4 ml.) of the crude reaction mixture was assayed for inhibitor as described previously. The estimate of the concentration of inhibitor was made in an approximate manner by assuming that L-tyrosinhydrazide functioned as a competitive inhibitor in this system. A value of v_0 = 0.653 × 10^{-4} M min.$^{-1}$ was obtained, from which K_S' = 0.148 M. The assumption of a value for K_I = 8.3 × 10^{-5} M gave [I] = 0.113 × 10^{-3} M, equal to a maximum conversion of 5.6% since the sum of the concentrations of L-tyrosinhydrazide and inhibitor equaled 0.002 M in the system being assayed.

Attempted Reaction of L-Tyrosine with Acethydrazide and α-Chymotrypsin.—Incubation of L-tyrosine with enzyme and acethydrazide at 25° for 1 hr. at pH 5.8 and at pH 7.9 did not result in the appearance of a species of low K_I, *i.e.*, 1-acetyl-2-(L-tyrosyl)-hydrazine.

Evaluation of 1-Acetyl-2-(L-tyrosyl)-hydrazine as an Inhibitor of the α-Chymotrypsin Catalyzed Hydrolysis of Methyl Acetyl-L-valinate.—The kinetic constants K_S' = $K_S(1 + [I]/K_I)$ and k_3' were determined in aqueous solutions at 25.0°, pH 7.90 and 0.10 M in sodium chloride with [E] = 0.1464 mg. protein-nitrogen per ml., [I] = 0.227 and 0.454 × 10^{-3} M and $[S]_0$ = 15 to 140 × 10^{-5} M. The inhibition was not totally competitive but was of a mixed type. The data were evaluated by a procedure to be described in a separate communication to give a value of K_I = 7.4 ± 0.3 × 10^{-5} M.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF WISCONSIN, MADISON 6, WISC., AND CHEMISCHES LABORATORIUM DER UNIVERSITÄT, FREIBURG I. BREISGAU, GERMANY]

The Effect of Solvent on Spectra. VII. The "Methyl Effect" in the Spectra of Dihydropyridines

BY DIETER HOFMANN, EDWARD M. KOSOWER[1a] AND KURT WALLENFELS

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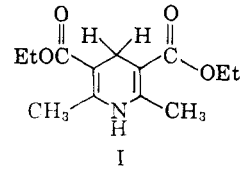
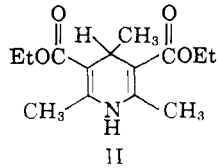
The puzzling difference in ultraviolet maximum between the "Hantzsch compounds," 2,6-dimethyl-3,5-dicarbethoxy-1,4-dihydropyridine (λ_{max} 3690 Å.) and 2,4,6-trimethyl-3,5-dicarbethoxy-1,4-dihydropyridine (λ_{max} 3490 Å.) is now explained as resulting from the relative increase in non-bonded repulsion between the carbethoxy group and the 4-methyl group in the excited state as compared with the compound lacking the 4-methyl group. The explanation is supported by the decrease in the effect when the carbethoxy groups are replaced by cyano groups in which the charge increment in the excited state is farther away from the 4-methyl group. The validity of the comparisons between the carbethoxy and cyano-substituted dihydropyridines is established by the parallelism in solvent effects upon the observed transitions. Data for the model compounds ethyl β-aminocrotonate and β-aminocrotononitrile are also reported.

The spectra of dihydropyridines are of considerable interest and importance because of the necessity for understanding in great detail the spectroscopic behavior of reduced diphosphopyridine nucleotide (DPNH).[1b] It was puzzling to find that replacement of one of the hydrogens at the 4-position in the "Hantzsch compound" 2,6-dimethyl-3,5-dicarbethoxy-1,4-dihydropyridine (I) with a methyl group (II) resulted in a shift of the absorption maximum to *shorter* wave lengths by 200 Å.[2] Since the methyl group is not attached directly to the conjugated system, the displacement of the maximum should have been small, all alkyl groups being approximately equivalent in their intrinsic electron-donating ability.[3,4] Examination of models of I and II indicated little serious steric hindrance in the ground state, but suggested the possibility that acquisition of charge by the oxygen in the carbethoxy group of the excited state (I* and II*) might be opposed by the methyl group on the 4-position.



I



II

The s-*trans* arrangement indicated in I and II (and I* and II*) is thought to be the most likely on the basis of the examination of models since there is appreciably more interference between the 2-

(1) (a) To whom requests for reprints should be addressed at Department of Chemistry, State University of New York, Long Island Center, Oyster Bay, N. Y. (b) The authors wish to acknowledge the generous support of the National Institutes of Allergy and Infectious Diseases through grant E-1608.

(2) This circumstance was called to our attention some years ago by Professor F. H. Westheimer, Harvard University.

(3) E. M. Kosower and J. A. Skorcz, *J. Am. Chem. Soc.*, **82**, 2195 (1960).

(4) A penetrating inquiry into the "Baker–Nathan order" for hyperconjugation, in which p-methyl groups on benzyl chloride systems are more effective than p-t-butyl groups in raising the rate over that for the unsubstituted system has been carried out by R. A. Clement, J. N. Naghizadeh and M. R. Rice, *ibid.*, **82**, 2449 (1960). Their results indicate that solvation of the solvolyzing molecule may differ sufficiently from the p-methyl case to the p-t-butyl that the observed rate in solution may indeed be slightly augmented for the former. However, approximate "intrinsic reactivities" (*i.e.*, corrected for solvation) are very close to being the same and, thus, intrinsic electron-supply by alkyl groups varies very little with the nature of the alkyl group, a conclusion in complete accord with that found for the effect of alkyl groups upon the position of the pyridinium iodide charge-transfer band.[5]