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The Non-enzymatic Cleavage of Peptide Bonds. II. The Chemical Cleavage of the Tryptophyl Bond in Several Synthetic Peptides[1,2]

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Several synthetic peptide derivatives containing tryptophan were examined for the selective cleavage at the tryptophyl residue, according to a new scheme involving ozonization and subsequent treatment with hydrazine. The ozonization reaction of the tryptophyl residue was carried out in the presence and in the absence of resorcinol in formic acid. Then the reaction of the ozonized product with hydrazine was performed at pH 3.6 to bring about the tryptophyl bond cleavage. The yield of the present cleavage reaction ranged from 35 to 68% on the basis of the starting peptide derivative used. The cleavage reaction could be followed spectroscopically in parallel with the exposure of the amino group acylated by the tryptophyl residue. The 2,3,4,5-tetrahydropyridazone derivative derived from the parental tryptophyl residue was identified by a comparison of both its chromatographic behavior and its ultraviolet spectrum with those of the standard sample. Thus it was confirmed that the present cleavage proceeded through the proposed reaction sequence.

In a previous paper, a new approach to effect the selective cleavage of tryptophyl bonds was proposed.[1] The cleavage reaction consists of two reactions; one is the oxidative conversion of the tryptophyl residue into a *N'*-formylkynurenyl residue (the first reaction), and the other is the selective cleavage of the tryptophyl bond by the reaction of the kynurenyl residue with hydrazine. The second step in the cleavage reaction was previously studied by using model compounds.[1] The reaction scheme is thought to be useful not only for the fragmentation of the peptide chain in the structural analysis of the protein molecule, but also for the study of the structure-function relationship of the biologically-active protein by the chemical modification of the particular tryptophyl residue, since the oxidized tryptophan in the protein can be easily characterized by identifying any newly-formed amino terminal amino acid after hydrazine treatment. In the present paper the authors will describe the cleavage reaction of the tryptophyl bond in several synthetic peptide derivatives according to the scheme depicted in Fig. 1.

Results and Discussion

Conversion of the Tryptophyl Residue into a *N'*-Formylkynurenyl Residue by Ozone. Among the oxidation reactions of indoles previously studied, such oxidants as ozone,[3,4] peracids,[5]

1) Part I of this series, M. Morishita, T. Sowa, F. Sakiyama and K. Narita, This Bulletin, **40**, 632 (1967). A part of this report was presented in this Bulletin, **40**, 433 (1967).

2) This investigation was aided in part by a grant from Tanabe Amino Acid Foundation.

3) B. Witkop, *Ann.*, **556**, 103 (1944).

4) A. Previero, E, Scoffone, C. A. Benassi and P. Pajetta, *Gazz. Chim. Ital.*, **93**, 849 (1963).

5) B. Witkop and H. Friedker, *Ann.*, **558**, 91 (1947).

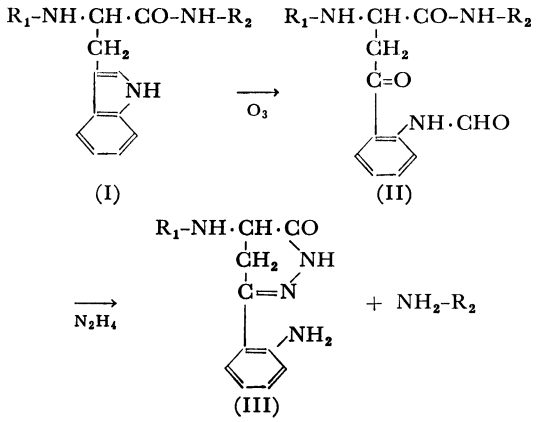


Fig. 1. The reaction sequence for the chemical cleavage of the tryptophyl bond.

and periodate ions[6] have been shown to effect the oxidative scission of the α,β-unsaturated bond of the pyrrole part in the indole nucleus. Molecular oxygen can also act as an oxidizing agent in either physiological[7] or photochemical[8] reactions. For the present purpose, the complete and selective oxidation of the tryptophyl residue in a peptide chain is desirable, and ozone was considered as one of the most favorable oxidants among the above agents. It has previously been reported that, on the controlled ozonization of protein amino acids, tryptophan could be selectively converted into *N'*-formylkynurenine with a side reaction, the oxidation of the sulfur atom of methionine.[9]

The reaction conditions for the quantitative conversion of tryptophan into *N'*-formylkynurenine was first examined since, in the experiment reported by Previero and Bordignon,[9] the amount of ozone to be used for the oxidation of tryptophan was not specified. The oxidation was followed by an increase in the absorption at 322 mμ which was characteristic of the chromophore of *N'*-formylkynurenine. Another determination of the oxidation product was simultaneously achieved by the ninhydrin assay of kynurenine after mild acid hydrolysis and subsequent chromatographic separation on filter paper.

The ozonization of tryptophan was very rapid, and the indole nucleus was oxidized almost completely within one minute either with or without resorcinol. As is shown in Fig. 2, the quantitative formation of kynurenine was observed by the ninhydrin assay when ozonization was carried out in a formic acid containing resorcinol (molar ratio of O_3 : resorcinol : tryptophan = 4 : 2 : 1). However, different features were observed when the same oxidation was monitored by the ultraviolet absorption (322 mμ, Fig. 3). In the presence of resorcinol, the ninhydrin method afforded more kynurenine than did the spectrometric method. It should be noted that no maximal formation of *N'*-formylanthranyl grouping was detected when kynurenine was quantitatively obtained. The same phenomenon was also observed on ozonization without resorcinol. This discrepancy can be explained by the formation of a certain

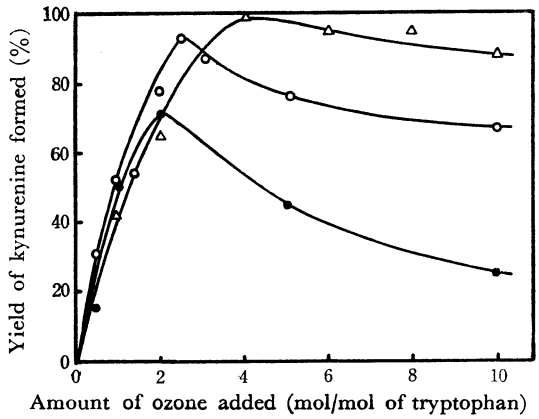


Fig. 2. Ozonization of tryptophan by various amounts of ozone (Formation of kynurenine after acid hydrolysis).
Reaction conditions: concentration of tryptophan was 5 mM in formic acid and ozone was added for a minute. Ozonization in the absence (-●-) or the presence (a molar equivalent (-○-) and twice equivalents (-△-) to the amino acid) of resorcinol was presented.

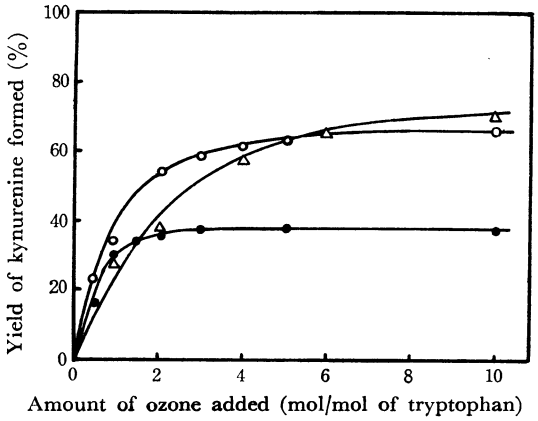


Fig. 3. Ozonization of tryptophan by various amounts of ozone (Formation of *N'*-formylkynurenine).
Reaction conditions and notations are similar with those described in Fig. 2. The amount of *N'*-formylkynurenine was estimated by absorbancy at 322 mμ.

6) L. J. Dolby and D. L. Booth, *J. Amer. Chem. Soc.*, **88**, 1649 (1965); M. Z. Atassi, *Arch. Biochem. Biophys.*, **120**, 56 (1967).
7) W. E. Knox and A. H. Mehler, *J. Biol. Chem.*, **187**, 149 (1950).
8) Z. Yoshida and M. Kato, *J. Amer. Chem. Soc.*, **76**, 311 (1954).
9) A. Previero and E. Bordignon, *Gazz. Chim. Ital.*, **94**, 630 (1964).

stable intermediate carrying no *N'*-formylanthranyl group; this intermediate was then transformed into kynurenine on acid hydrolysis. Resorcinol was obviously effective in enhancing the ozonization yield of tryptophan and in reducing the over-oxidation of the ozonization products. Phloroglucinol was also effective, but tyrosine was not.

When the carbobenzoxy derivative of tryptophan was ozonized under the best conditions described above, no quantitative formation of kynurenine was observed. In accounting for this phenomenon it was postulated that the bulky aromatic group attached to the tryptophan residue might interfere with the access of ozone to the indole nucleus when the oxidant was added to the reaction media over a short period. Thus, the ozonization of *N*-α-carbobenzoxytryptophylleucine was examined from time to time by both the ninhydrin and spectrometric methods (Fig. 4). The increase in the absorption at 322 mμ reached a plateau after one minute, but the amount of kynurenine estimated by the ninhydrin reaction gradually increased and an almost quantitative formation was observed after half an hour. Such a slow addition of ozone was also recommended by Previero *et al.*[4,9] for the effective conversion of tryptophan to *N'*-formylkynurenine.

Under the same conditions, the ozonization of the tryptophyl residue in several synthetic oligopeptides was undertaken. As is shown in Table 1, on the ozonization of the peptide in the presence of resorcinol the tryptophyl residue could be oxidized to the *N'*-formylkynurenyl residue in a 51—100% yield and, on acid treatment, the formation of kynurenine was estimated to be 80—99%. As long as ozonization is utilized for the first step in the cleavage reaction, it is not relevant

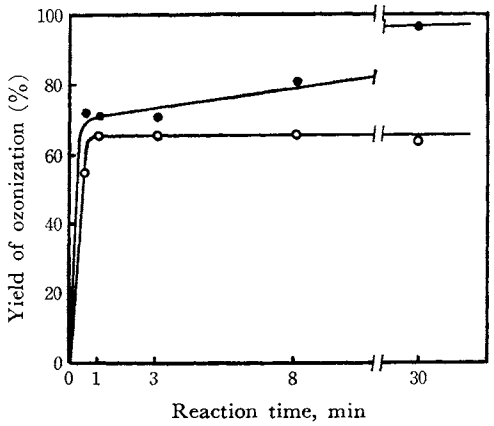


Fig. 4. Ozonization of carbobenzoxytryptophylleucine.
Reaction conditions: concentrations of tryptophan and resorcinol were 5 mM and 10 mM, respectively. Ozone (1—2% in oxygen, total amount was four-fold molar equivalents to tryptophan) was bubbled for half an hour. The extent of ozonization was estimated by both absorbancy at 322 mμ (-○-) and determination of kynurenine after acid hydrolysis (-●-).

to estimate the oxidation of the tryptophyl residue only by absorption at 322 mμ since the *N'*-formylkynurenine would not be a single ozonization product. However, since the second step in the cleavage reaction was performed in weakly acid media, the intermediate ozonization product of the tryptophyl residue can be expected to be readily hydrolyzed to kynurenine during heating in the aqueous media. For the present chemical cleavage of the tryptophyl bond, the carbonyl function at the γ-position is indispensable and the kynurenyl

TABLE 1. OZONIZATION OF THE TRYPTOPHYL PEPTIDE DERIVATIVE

	Yield of kynurenine formed (%)			
	in the absence of resorcinol		in the presence of resorcinol	
	UV-absorption*	Kynurenine**	UV-absorption*	Kynurenine**
Z-Trp-Gly-OH	51	85	51	88
Z-Trp-Ala-OH	55	83	60	92
Z-Trp-Leu-OH	51	88	55	99
Z-Trp-Phe-OH	54	88	60	96
Z-Trp-Asp-OH	49	77	63	80
Z-Ala-Trp-Leu-OH			60	81
H-Ala-Trp-Leu-OH	46	80	107	96
H-Gly-Trp-Gly-OH	55	75	103	85
H-His-Phe-Arg-Trp-Gly-OH	49		83	87
H-His-Phe-Lys-Trp-Gly-OH			83	82
H-His-Phe-Orn-Trp-Gly-OH			86	86
H-Phe-Arg-Trp-Gly-Ser-Pro-Pro-OH			95	

Z: Carbobenzoxy
* determined as *N'*-formylkynurenine by the absorption at 322 mμ.
** determined by the ninhydrin reaction after acid hydrolysis.

residue is a potent candidate as well as its *N'*-formyl derivative in the second step.[10] Therefore, photooxidation would be another promising reaction for the quantitative, selective conversion of tryptophan into a kynurenine derivative.[11]

Reaction of the Ozonized Product of the Tryptophyl Peptide with Hydrazine (the Second Step). *N*-α-Carbobenzoxytryptophylleucine oxidized by ozone was reacted with hydrazine under the conditions described previously.[1] The formation of the *N'*-formylkynurenyl residue was moderately achieved (66% yield), and about a half of the *N'*-formylkynurenyl bond could be cleaved. The leucine was released in a 31% yield, which was calculated on the basis of the parental tryptophyl peptide. In order to establish more effective conditions for the release of leucine, the reaction of the ozonized tryptophyl peptide with hydrazine was investigated further. The modification of certain parameters usually favorable for the present type of chemical reaction, that is, an increase in the reaction temperature and the reaction period, was not preferable for the selective cleavage reaction of the peptide bond. The effect of the concentration of hydrazine on the release of leucine was first examined. As is shown in Fig. 5, a five-fold molar excess of hydrazine was insufficient for the efficient cleavage reaction of the *N'*-formylkynurenyl bond and the maximal liberation of the amino acid (quantitative from the *N'*-formylkynurenyl residue formed) attained when twenty or more equivalent of the binucleophile were used. Thus, several of the tryptophyl peptides oxidized were submitted to the cleavage reaction: the amino group linked originally with the carboxyl group of the tryptophyl residue could thus obtained in a 35—68% yield (Table 2). The cleavage yield apparently depended on the amount of *N'*-formylkynurenine formed. As the last column in Table 2 shows, the tryptophyl peptide bond was partly hydrolyzed in certain cases when the parental derivative was reacted with hydrazine without ozonization.

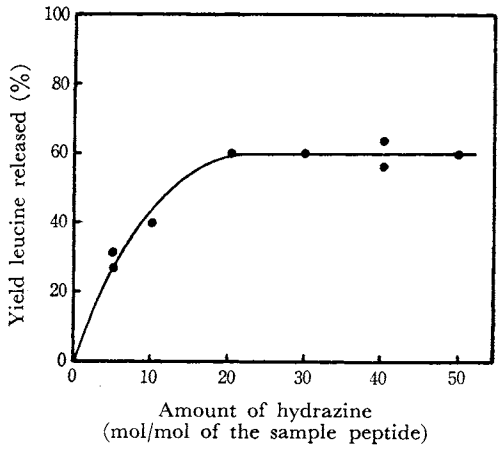


Fig. 5. Effect of concentration of hydrazine on release of leucine from the oxidized product of carbobenzoxytryptophylleucine.

Then the pH-dependence of the cleavage reaction of the *N'*-formylkynurenyl bond was again examined in order to minimize such undesirable non-specific hydrolysis. The release of the amino group was not significantly affected by the concentration of hydrogen ions between pH 3.6 and pH 5.85 (Fig. 6). Furthermore, no remarkable difference in the rate of the cleavage reaction was observed at the more extreme pH's. Therefore, it is expected that the hydrazine treatment at pH 5.85 would give a fruitful result since the non-specific hydrolysis of the peptide bond may occur to a lesser extent at this pH.

Table 2. Yield of the specific cleavage at the tryptophyl bond*

Starting peptide derivative	Released amino acid	Released amino acid by the specific cleavage (%)		Non-specific hydrolysis
		oxidized in the absence of resorcinol	oxidized in the presence of resorcinol	
Z-Trp-Gly-OH	Glycine	22	35	7
Z-Trp-Ala-OH	Alanine	47	57	5
Z-Trp-Leu-OH	Leucine	41	68	0
Z-Trp-Phe-OH	Phenylalanine	31	49	0
Z-Trp-Asp-OH	Aspartic acid	41	52	13
Z-Ala-Trp-Leu-OH	Leucine	—	54	—
H-His-Phe-Arg-Trp-Gly-OH	Glycine	—	45	6
H-His-Phe-Lys-Trp-Gly-OH	Glycine	—	43	7
H-His-Phe-Orn-Trp-Gly-OH	Glycine	—	49	12

* Calculated on the basis of the amount of the starting tryptophyl peptide used.

10) F. Sakiyama, unpublished data.
11) C. A. Benassi, E. Scoffone, G. Galiazzo and G. Jori, *Photochem. Photobiol.*, **6**, 857 (1967).

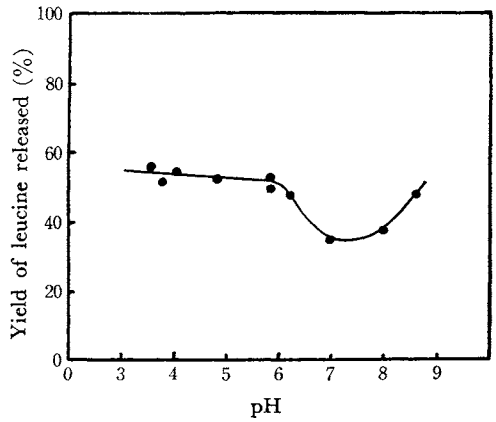


Fig. 6. pH-Profile of release of leucine from the oxidized product of carbobenzoxyalanyltryptophylleucine.

It should be possible to identify the counterpart of the newly-exposed amino group by a conventional technique. The other reaction product which was derived from the tryptophyl residue is probably 4-peptidylamino-6-(*o*-aminophenyl)-2,3,4,5-tetrahydropyridaz-3-one (III). However, little has been known about the chemical properties of the 4-amino-6-(*o*-aminophenyl)-2,3,4,5-tetrahydropyridaz-3-one (IV) derivative. Therefore, two 4-acylamino derivatives of IV, the 4-carbobenzoxy (V) and 4-*N*-carbobenzoxy-L-alanyl (VI) derivatives, were prepared by the reaction of the corresponding α-acylamino-γ-oxo acid ester with hydrazine. Both pyridazones developed an orange color with the Ehrlich reagent, emitted a yellow-orange fluorescence when excited at 260 mμ and showed a characteristic absorption near 350 mμ; this absorption was attributed to a conjugated system composed of the *o*-aminophenyl group and the ketimino function in the tetrahydropyridazone ring.

Then the cleavage reaction of the *N'*-formylkynurenyl bond was spectrophotometrically monitored (Fig. 7). The absorption at 322 mμ due to *N'*-formylkynurenine decreased rapidly in the early stage of the reaction and a new absorption maximum emerged near 350 mμ. This new absorption maximum, however, was gradually shifted to a shorter wavelength after about two hours. In this case the substrate was the carbobenzoxy derivative of tryptophylleucine, and the release of leucine, as expected, was observed in parallel with the increase in the absorption at 350 mμ until the amount of leucine reached a plateau (Fig. 8). However, it has to be noted that the formation of the tetrahydropyridazone derivative was estimated to be less than the concomitant release of leucine throughout the reaction period. For this reason, it was supposed, from the instability of III, that the compound (III), once formed, tended to decompose during the cleavage reaction.

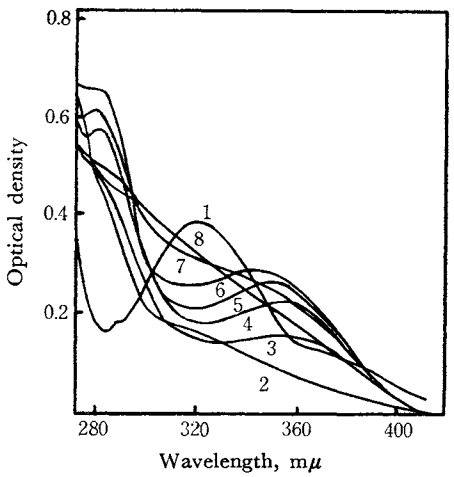


Fig. 7. Change of absorption spectrum during the reaction of the oxidized carbobenzoxytryptophylleucine with hydrazine in a 50% ethanol solution.
1: oxidation product of carbobenzoxytryptophylleucine, 2: after 20 min, 3: after 40 min, 4: after 1 hr, 5: after 1.5 hr, 6: after 2.5 hr, 7: after 3 hr, 8: after 3.5 hr.

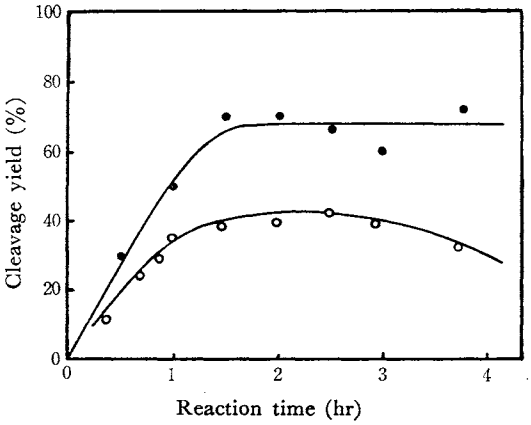


Fig. 8. Relationship between the change of absorbancy at 350 mμ (-○-) and release of leucine (-●-) during the reaction of the oxidized carbobenzoxytryptophylleucine with hydrazine.

By the thin-layer chromatography of the reaction mixture after the reaction of the ozonized carbobenzoxyalanyltryptophylleucine for two hours, two new yellow substances were detected. The major one, slowly moving, was identified as VI, and the other, a minor component, was assumed to be one of decomposition products since this substance showed a spectrum quite different from that of VI. This product was neither alanine nor leucine, and it gave no ninhydrin reaction even after acid hydrolysis.[12] Though no details on the decomposition reaction of VI are available at the present time, it may be concluded that the cleavage reaction of the tryptophyl bond occurred through the series of chemical reactions presented in Fig. 1.

Experimental

Methods and Materials. Paper chromatography on Toyo No. 51 filter paper was carried out using a solvent system, A (*n*-butanol-water-acetic acid, 4 : 1 : 1 by volume) or B (pyridine-*n*-butanol-water-acetic acid, 10 : 15 : 12 : 3 by volume). Thin-layer chromatography (silica gel G, Merck AG) was also performed with either solvent system. The amino acid was determined by Cd-ninhydrin reaction after chromatography on filter paper.[13] The absorption spectra were measured by a Beckmann spectrometer, type DB. Ozone (1—2%) was generated from oxygen by a generator manufactured by the Nippon Ozone Co., Ltd., type 0—3—2, and the ozone concentration was determined by the KI-$Na_2S_2O_3$ method. The formic acid (99—100%) was purchased from Merck AG and was used without purification. Carbobenzoxy-DL-tryptophyl-L-alanine, -L-leucine, -L-phenylalanine, and -L-aspartic acid were prepared by a conventional method. Four tryptophyl peptides related to the adrenocorticotropic hormone were courteously supplied by Dr. H. Yajima of Kyoto University, Kyoto. All the melting points were uncorrected.

Ozonization of Tryptophan with or without Resorcinol. Into 1.0 m*l* of a formic acid solution containing 5 μmol of tryptophan and resorcinol (0, 5 or 10 μmol), the desired amount of ozone was bubbled within one minute at room temperature. Aliquots (0.1 m*l*) of the reaction mixture were then transferred into 3 m*l* of ethanol, and the absorbancies at 322 and 280 mμ were measured. The yield of *N'*-formylkynurenine formed was estimated by the absorbance at 322 mμ on the basis of the molar extinction of *N*-acetyl-*N'*-formylkynurenine ($\varepsilon = 4.25 \times 10^3$ in the mixture of formic acid and ehtanol, 1 : 10 by volume). Another aliquot (0.1 m*l*) was dried up under reduced pressure and incubated in 0.05 m*l* of 1 N hydrochloric acid for one hour at room temperature in order to remove the *N'*-formyl group. Then 10 μ*l* of the above solution was submitted to paper chromatography with either the A solvent or with water in order to determine kynurenine.

Ozonization of Several Synthetic Peptide with Ozone. One milliliter of a formic acid solution containing 5 μmol of the tryptophyl peptide or its derivative was treated by a stream of ozone (20 μmol with, or 10 μmol without, 10 μmol of resorcinol) for half an hour at room temperature. The amount of *N'*-formylkynurenine and kynurenine were estimated much as has been described above except for the procedure of the hydrolysis of kynurenine determination: that is, the hydrolysis of the ozonized peptide derivative was carried out under more drastic conditions (105°C for 20 hr with 6 N hydrochloric acid).

Table 3. Buffers used for the cleavage of the peptide bond of Z-DL-Trp-L-Leu-OH in various concentrations of hydrazine

pH	Final concn. of hydrazine (M)	Final concn. of acetic acid (M)
2.65	0.0	2.0
3.60	0.1	2.0
3.60	0.2	4.0
3.60	0.3	6.0
3.60	0.4	8.0
3.60	0.5	9.0

Effect of the Concentration of Hydrazine and the pH on the Release of Leucine from the Oxidized Product of *N*-α-Carbobenzoxytryptophyl Peptide. Into 10 m*l* of formic acid containing *N*-α-carbobenzoxy-DL-tryptophyl-L-leucine (45.1 mg, 100 μmol), 200 μmol of ozone were bubbled at room temperature within one minute. Aliquots (0.1 m*l*) were taken out and the formic acid was removed under reduced pressure. Into the residue, 0.5 m*l* of the hydrazine-acetate buffer listed in Table 3 was added. The solution or suspension was then heated to boil for two hours. When ten microliters of the reaction mixture were chromatographed on filter paper with the A solvent, the leucine was identified and determined.

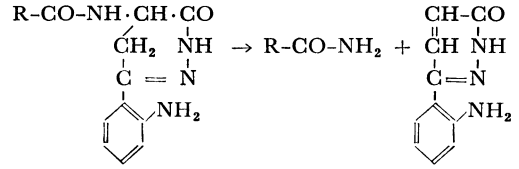
For the study of the pH dependence of the hydrazine treatment, the oxidized *N*-α-carbobenzoxy-L-alanyl-L-tryptophyl-L-leucine was similarly treated with a hydrazine-acetate buffer at various pH values (molar ratio of the peptide derivative to hydrazine = 1 : 20 in all experiments). (*Cf.* Table 4).

Cleavage Reaction of the Tryptophyl Bond by a Standard Procedure. One milliliter of a formic acid solution containing 5 μmol of the sample peptide derivative was treated with 20 or 10 μmol of ozone for half an hour with or without resorcinol (10 μmol). An aliquot (0.1 m*l*) taken out was evaporated to dryness and treated with a hydrazine(0.1 M)-acetate buffer (pH 3.6) at 105°C for 2 hr in a sealed tube. Then the sample was transferred quantitatively onto filter paper for chromatographic identification, and the amount of amino acid released was determined. A duplicate

Table 4. Buffers used for the cleavage of the tryptophyl bond of Z-L-Ala-L-Trp-L-Leu-OH at various pH's

pH	Final concn. of hydrazine (M)	Final concn. of acetic acid (M)
3.60	0.2	2.0
3.75	0.2	1.0
4.10	0.2	0.5
4.85	0.2	0.28
5.85	0.2	0.175
6.20	0.2	0.17
6.60	0.2	0.165
7.00	0.2	0.16
8.00	0.2	0.11
8.70	0.2	0.00

12) Following reaction was considered as one of possible decompositions of 4-acylaminotetrahydropyridazone.



13) R. C. Canfield and C. B. Anfinsen, "The Proteins," Vol. 1, ed. by H. Neurath, Academic Press, New York (1963), p. 318.

experiment was made for the cleavage reaction of each tryptophyl peptide derivative.

The non-specific hydrolysis of *N*-α-carbobenzoxy-tryptophyl amino acids was studied by the same procedure except for the ozonization.

N-α-Acetyl-N'-formyl-DL-kynurenine. *N*-α-Acetyl-DL-tryptophan (2.46 g) dissolved in 100 m*l* of formic acid was oxidized with a stream of ozone at room temperature until the absorption at 280 mμ disappeared almost completely. On the evaporation of the formic acid, the resulting oily product was treated with methanol, and the slightly yellow powder (1.25 g) thus obtained was crystallized from methanol. Mp 194°C. λ_{max} 322 mμ ($\varepsilon=4.25\times10^3$ in ethanol and 4.05×10^3 in ethanol-formic acid (30 : 1 by volume)). ν(Nujol) 3395, 3300, 1729, 1710, 1590 and 1559 cm^{-1}. R_f(A) 0.81 (orange with an Ehrlich reagent).

Found: C, 56.16; H, 5,13; N, 9.74%. Calcd for $C_{13}H_{14}O_5N_2$: C, 56.11; H, 5.07; N, 10.07%.

4-L-(N-carbobenzoxy-L-alanylamino)-6-(o-aminophenyl)-2,3,4,5-tetrahydropyridaz-3-one (VI). By a similar procedure, the *N*-α-carbobenzoxy-L-alanyl-tryptophan methyl ester (400 mg) was oxidized by ozone. After the removal of the formic acid, the oily residue was dissolved in 10 m*l* of ethanol. To the ethanol solution 0.125 m*l* of 80% hydrazine hydrate was added, after which the solution was allowed to stand overnight at room temperature. The slightly yellow crystals thus separated were collected and recrystallized from ethanol (377 mg, 92%). Mp 197.5—198.5°C. R_f(B) 0.87 (orange with an Ehrlich reagent and a yellowish-orange fluorescence under ultraviolet light). λ_{max} 350 mμ ($\varepsilon=8.13\times10^3$), 285 mμ ($\varepsilon=10.5\times10^3$) and 250 mμ ($\varepsilon=16.5\times10^3$) in ethanol. ν(Nujol) 3420, 3270, 1660, 1609, 1558, 1540, 1530 and 1488 cm^{-1}.

Found: C, 61.19; H, 5.46; N, 16.61%. Calcd for $C_{21}H_{23}O_4N_5$: C, 61.60; H, 5.66; N, 17.11%.

4-L-Carbobenzoxyamino-6-(o-aminophenyl)-2,3,4,5-tetrahydropyridaz-3-one (V). By a similar procedure, V was prepared from the *N*-α-carbobenzoxy-L-tryptophan methyl ester. The crude product was recrystallized from methanol. Yield, 37%. Mp 199—200°C. R_f(B) 0.95 and (A) 0.81 (orange with an Ehrlich reagent and a yellowish-orange fluorescence under ultraviolet light). λ_{max} 350 mμ ($\varepsilon=8.10\times10^3$), 285 mμ ($\varepsilon=11.4\times10^3$) and 250 mμ ($\varepsilon=16.0\times10^3$) in ethanol. ν(Nujol) 3450, 3285, 3200, 1609, 1600, 1588, 1544, 1527 and 1488 cm^{-1}.

Found: C, 63.39; H, 5.25; N, 16.10%. Calcd for $C_{18}H_{18}O_3N_4$: C, 63.89; H, 5.36; N, 16.56%.

6-(o-Aminophenyl)-2,3,4,5-tetrahydropyridaz-3-one. This compound was prepared from β-indolepropionic acid in a 15% yield by ozonization and subsequent hydrazine treatment. λ_{max} 350 mμ ($\varepsilon=7.0\times10^3$), 285 mμ ($\varepsilon=10.6\times10^3$) and 250 mμ ($\varepsilon=15.9\times10^3$) in ethanol.

Found: C, 63.26; H, 5.84; N, 21.74%. Calcd for $C_{12}H_{11}ON_3$: C, 63.47; H, 5.84; N, 22.21%.

N-α-Carbobenzoxy-L-alanyl-L-tryptophyl-L-leucine Methyl Ester. *N*-α-Carbobenzoxy-L-alanine was coupled with the L-tryptophyl-L-leucine methyl ester by dicyclohexylcarbodiimide, and the pure, protected tripeptide ester was then obtained by recrystallization from ethyl acetate-petroleum ether. Mp 169.5—170°C. $[\alpha]_D^{26}=-34.5$ (*c* 1.0, glacial acetic acid).

Found: C, 64.74; H, 6.70; N, 10.32%. Calcd for $C_{29}H_{36}O_6N_4$: C, 64.91; H, 6.76; N, 10.44%.

N-α-Carbobenzoxy-L-alanyl-L-tryptophyl-L-leucine. The *N*-α-carbobenzoxy-L-alanyl-L-tryptophyl-L-leucine methyl ester (1.07 g) was suspended in methanol (30 m*l*), and the 2 N sodium hydroxide (2.2 m*l*) was added. The alkaline solution was stirred for an hour under cooling with ice water and then for four hours at room temperature. After the evaporation of the methanol, the aqueous solution was acidified with 1 N hydrochloric acid, and the white precipitates thus separated was collected. Yield, 1.01 g (96.2%). The crude protected tripeptide was dissolved in ethyl acetate (30 m*l*); by the addition of dicyclohexylamine (0.48 m*l*), the dicyclohexylammonium salt (1.30 g, 92.2%) was obtained. Mp 183—185°C.

Found: C, 66.81; H, 8.36; N, 9.95%. Calcd for $C_{40}H_{57}O_6N_5\cdot H_2O$: C, 66.55; H, 8.24; N, 9.70%.

The dicyclohexylammonium salt (1.30 g) suspended in water (50 m*l*) was acidified with 1N hydrochloric acid (2 m*l*), and the white precipitates were extracted into ethyl acetate, which was then dried over anhydrous sodium sulfate and evaporated. Since the protected tripeptide was not crystallized, it was again dissolved in a 5% sodium hydrogencarbonate solution. The pure tripeptide was then separated by acidification with 1N hydrochloric acid. Yield, 0.98 g (96.9%). Mp 156—157°C. $[\alpha]_D^{26}=-29.5$ (*c* 1.0, glacial acetic acid).

Found: C, 64.29; H, 6.52; N, 10.46%. Calcd for $C_{28}H_{34}O_6N_4$: C, 64.35; H, 6.56; N, 10.72%.

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