Preferential Hydrolysis of Kynurenine Peptides

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The preferential cleavage of kynurenine peptide bonds was studied through reduction to \( \gamma \)-(o-aminophenyl)-homoserine derivatives followed by mild acidic as well as mild alkaline hydrolysis. The reduction of keto group of kynurenine to hydroxy one was achieved by controlled potential electrolysis at \(-1.05\) Volts. In order to understand the mechanism of hydrolysis some model compounds related to kynurenine peptides were also synthesized and studied. Hydrolysis of \( \gamma \)-(o-aminophenyl)-homoserine peptides in acidic media is related only to the assistance of \( \gamma \)-hydroxy function, whereas in alkaline media also aromatic amino group is involved.

The conversion of tryptophan to kynurenine was extensively investigated in our laboratories through performic acid oxidation and, with better results, in peptides and proteins by ozonization and dye sensitized photooxidation with the aim to reach a preferential chemical cleavage at the tryptophan residue. The preparation of kynurenine peptides was firstly achieved from the corresponding tryptophan peptides and modified lysozyme was obtained and studied.

In this paper we present results about the preferential hydrolysis of kynurenine peptides through reduction by controlled potential electrolysis of the keto group to the hydroxy followed by mild acidic or basic hydrolysis.

In addition some kynetic studies on the cleavage of selectively reduced kynurenine peptides and of related derivatives are reported.

Selective reduction of the keto group of kynurenine (1) to hydroxy group of \( \gamma \)-(o-aminophenyl)-homoserine (2) was previously studied polarographically by one of us and found to occur over a large interval of pH with the consumption of two electrons:

![Diagram]

Table I. Polarographic Reduction: Half-Wave Potentials of Kynurenine and Kynurenine Peptides in 5% and 50% Acetic Acid.

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<tr>
<th>Compounds</th>
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<td>CH(_3)COOH 5%</td>
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<td>Kynurenine</td>
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These studies, already accomplished with kynurenine alone, have now been extended to some kynurenine peptides in 5% and 50% acetic acid.

As reported in Table I the examined kynurenine derivatives present similar half-wave potentials of polarographic reduction as that done by kynurenine itself. Some variations among the compounds could depend on the different molecular structure.

Controlled potential electrolysis may be better accomplished at \(-1.05\) Volts vs. SCE, the reduction of keto group being controlled by the lowering of current flow and also checked by recording polarographic of \( \gamma \)-(o-aminophenyl)-homoserine peptides present similar half-wave potentials of polarographic reduction as that done by kynurenine itself. Some variations among the compounds could depend on the different molecular structure.

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grams without changing the experimental conditions\(^9\). Reduction is accompanied by disappearance of the absorption maximum at 360 m\(\mu\), whereas a new maximum is noted at 280 m\(\mu\) (see Fig. 1) with a contemporaneous decrease of fluorescence under UV light.

![Absorption spectra of kynurenine and \(\gamma\)-\((o\text{-aminophenyl})\)-homoserine](image)

**Fig. 1.** Absorption spectra of kynurenine (1) (-----) and \(\gamma\)-\((o\text{-aminophenyl})\)-homoserine (2) (-------) in phosphate buffer 0.05 M, pH 6.

Furthermore, reduced compounds [\(\gamma\)-\((o\text{-aminophenyl})\)-homoserine derivatives] show on paper and TLC chromatograms a bright yellow reaction with p-dimethylaminobenzaldehyde reagent.

Controlled potential electrolysis appears a very selective method of reduction since no other amino acid is affected with the exception of cystine which is quantitatively converted to cysteine\(^10\). Fig. 2 shows a pattern of polarographic reduction of kynurenine in 5% acetic acid.

Kynurenine and its peptides may alternatively be reduced to \(\gamma\)-\((o\text{-aminophenyl})\)-homoserine with NaBH\(_4\)\(^11\) but this method is less accurate than the electrochemical one, since over reduction may result, and pure products are obtained only after crystallization.

**Fig. 2.** Pattern of polarographic reduction of kynurenine in 5% acetic acid.

For these reasons the electrochemical reduction is preferable, especially when small amounts of substance are available.

In order to study the mechanism of cleavage some protected kynurenine peptides were prepared\(^9\) and the carbobenzoxy group was removed by hydrogenolysis. Their melting points, rotatory power, chromatographic behaviour and analytical data are reported in Table II.

All these kynurenine peptides were reduced to the corresponding \(\gamma\)-\((o\text{-aminophenyl})\)-homoserine derivatives and then hydrolyzed in mild acide (0.2 N HCl) as well as in mild alkaline conditions (0.5 M NaHCO\(_3\)) in a sealed evacuated tube at 100\(^\circ\).

The amino acids released from reduced derivatives at various times of hydrolysis were determined by the amino acid analyzer. Fig. 3a shows the rate of liberation of the N-terminal or C-terminal amino acid from the \(\gamma\)-\((o\text{-aminophenyl})\)-homoserine peptides during acidic hydrolysis. The results obtained with \(\gamma\)-\((o\text{-aminophenyl})\)-homoseryl-glycine ethyl ester (3), phenylalanyl-\(\gamma\)-\((o\text{-aminophenyl})\)-homoserine methyl ester (4), and alanyl-\(\gamma\)-\((o\text{-aminophenyl})\)-homoseryl-glycine ethyl ester (5) are here tabulated. Comparable data have been also obtained with other model compounds as alanyl-\(\gamma\)-\((o\text{-aminophenyl})\)-homoserine methyl ester, carbobenzoxy-\(\gamma\)-\((o-}


\(^{10}\) G. Markus, Federat. Proc. 19, 340 [1960].

aminophenyl)-homoserine, glycine ethyl ester and carbobenzoxy-phenylalanyl-γ-(o-aminophenyl)-homoserine methyl ester.

The obtained results show that the γ-hydroxy group labilizes both peptide bonds, but in a different extent. In fact the N-terminal amino acid is released more rapidly than the C-terminal one.

In order to ascertain if only the hydroxy group is involved in the preferential cleavage of γ-(o-aminophenyl)-homoserine peptides, some model compounds lacking of the ortho amino group have been studied. Thus we have prepared alanyl-γ-phenyl-homoserine glycine ethyl ester (6) and in addition glyceryl-γ-phenyl-hydroxy-propylamide (7) and γ-phenyl-γ-hydroxy-butryryl glycine ethyl ester (8), in which not only aromatic amino group is missing.
but also the z-carboxy and z-amino functions are respectively absent.

These derivatives were subjected to mild acidic hydrolysis and the rate of cleavage of peptide bonds, determined on the basis of released amino acids, are reported in Fig. 3 b.

A comparison of the results reported in Figs. 3 a, 6 and 3 b shows that there is no appreciable difference in the rate of acidic hydrolysis of both peptide bonds of the different compounds.

These results point out that the aromatic amino group is not involved in the acidic hydrolysis of reduced kynurenine peptides. Moreover neither z-carboxy, nor z-amino group have any influence on the hydrolysis of the near peptide bond.

These data suggest the following pathway for the acidic hydrolysis of C-peptide bond of γ-(o-aminophenyl)-homoserine and γ-phenyl-homoserine peptides:

This mechanism is analogous to that demonstrated by BRUCE and MARQUARDT\(^\text{12}\) in the acidic hydrolysis of γ-hydroxy-butryramide.

On the other hand, the cleavage of the N-peptide bond of γ-(o-aminophenyl)-homoserine peptides might be explained on the basis of a "N→O acil migration" as postulated by ZAHN and ZÜRN\(^\text{13}\) for the acidic hydrolysis of hydroxyllysyl peptides and also by SANGER and TUPPY\(^\text{14}\) in the hydrolysis of serine and threonine peptides.

Considering now the effect of basic medium, also in this case both peptides bonds of γ-(o-aminophenyl)-homoserine are splitted, in agreement with the results of PREVIERO et al\(^\text{11}\).

Fig. 4 a shows the rate of cleavage of γ-(o-aminophenyl)-homoserine peptides, while Fig. 4 b reports the behaviour of alanyl-γ-phenyl-homoserine-glycine ethyl ester (6), glycyl-γ-phenyl-γ-hydroxy-propylamide (7) and γ-phenyl-γ-hydroxy-butryl-glycine ethyl ester (8).

It is noteworthy that a) no hydrolysis of the N-terminal amino acid bond takes place when the aromatic amino group of γ-(o-aminophenyl)-homoserine is lacking, and b) the rate of hydrolysis of the C-terminal amino acid bond is decreased.

These results prove that the aromatic amino group of reduced kynurenine is involved in the alkaline hydrolysis. In our opinion this group does not act, however, attacking the peptide bonds since it would occur through a very unlike eight or seven-member ring.

To explain the role of the aromatic amino group in the hydrolysis it is tentatively proposed the formation of a hydrogen bond with the γ-hydroxy group, with the result to increase the basicity of this last.

In order to control the validity of this hypothesis the I.R. spectra of 2-methyl-o-aminophenyl-carbinol (9) and 2-methyl-phenyl-carbinol (10) were compared.
IR investigations of a 0.001 M solution in carbon tetrachloride give evidence for an intramolecular hydrogen bond between amino and hydroxy group as appears from observed ν OH-frequencies (3600 and 3680 cm⁻¹ respectively).

The intramolecular participation of amino and hydroxy group in alkaline hydrolysis of γ-(o-aminophenyl)-homoserine peptide bonds may than be depicted as follows:

As shown in Fig. 4 this participation of the ortho amino group is a prerequisite for the cleavage of the N-terminal bond of γ-(o-aminophenyl)-homoserine peptides, whereas in the cleavage of the C-peptide bond it probably acts also increasing the reaction rate.

In this regard we remember that in alkaline media not only the C-terminal bond of γ-phenyl-homoserine peptide is cleaved (see above) but also amide bond of γ-hydroxy-butryramide (see BRUCE and MARQUARDT 12).

The participation of aromatic amino group of γ-(o-aminophenyl)-homoserine by hydrogen bond coordination seems to play an assistance to the catalysis similar to that observed in some enzymes 15.

**Experimental**

**Materials.** — β-Benzoyl propionic acid 16, β-benzoyl ethylamine 17 and 2-amino-β-benzoyl-propionic acid 18 were prepared according to literature. This last compound was also prepared from acetophenone according to a new procedure below reported. 2-Methyl o-aminophenyl carbinal and 2-methyl phenyl carbinal were prepared respectively from o-nitrocetophenon and acetophenon 19. The substances for these synthesis were Fluka products (Fluka AG, Basle, Switzerland). All other reagents, when not otherwise specified, were Merck reagent grade products (Merck AG, Darmstadt, Germany).

**Methods.** — UV spectra were obtained with a Beckman Model DB spectrophotometer connected to a Sargent recorder. Absorptions at single wavelengths were determined with a Hilger Uvispek Model H.700 spectrophotometer. The IR spectra were obtained with a Perkin-Elmer Model 337 spectrophotometer. The pH was determined with a Beckman expanded scale pH-meter, Model 76. The rotatory powers were measured with a Perkin-Elmer Model 141 polarimeter; concentrations are given in grams per 100 ml of solvent. The melting points were determined on a Tottoli apparatus (Büchi) and are uncorrected.

For polarographic reduction and for controlled cathodic potential electrolysis an AMEL Model 557/SV potentiostat was used. Polargraphic cells were AMEL type. Temperature was 28 ± 0.1. Oxygen was removed with nitrogen bubbling for 15 minutes. In the case of controlled potential electrolysis of kynurenine peptides, the sample (10 – 20 mg) was dissolved in 10 ml of 5% acetic acid and a current of −1.05 Volts vs. SCE was employed; polarograms were recorded at the beginning and sometimes during the course of the reduction. Reduction of kynurenine peptides was considered ended at the disappearance of the characteristic reduction wave of its keto group (E 1/2 ≥ −1.0 Volts, see Table I).

Amino Acid Analyzer was a Technicon apparatus (Technicon Instruments Company Ltd, Chertsey, England).

Thin layer chromatography (SiO2) was performed using the following solvent mixtures: n-butyl alcohol: water: glacial acetic acid (3:1:1) (Rf1); ethyl acetate: pyridine: water: glacial acetic acid (60:20:14:6) (Rf2). The compounds were in turn detected by ninhydrin spray, hypochlorite test 20 and reaction with p-dimethylaminobenzaldehyde.

**Kynurenine peptides.** — The N-benzoxycarbonyl group of protected kynurenine peptides, prepared according our procedure 2, was removed by hydrolysis in the presence of palladium on charcoal as a catalyst in methanol or ethanol. The catalyst was removed and the solvent evaporated in vacuo. The residual oil, after addition of an equimolecular amount of dry HCl in ethanol, was crystallized from various solvents (see Table II).

β-Benzoyl-propionyl-p-nitrophenyl ester. — To a solution of 3.56 g (20 mmoles) of β-benzoyl propionic acid, 2.78 g (20 mmoles) of p-nitrophenol in 100 ml of anhydrous chloroform, cooled to 0°C, 4.12 g (20 mmoles) of dicyclohexylcarbodiimide were added. After 30 minutes at 0°C and 24 hrs at room temperature, the precipitate of dicyclohexylurea was filtered off and washed with chloroform. After washings with 5% NaHCO3 and water, the solution was evaporated to dryness and the residue crystallized from methanol-ethyl ether. The yield was 4.78 g (80%), mp 124°.


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Anal. Calcd. for C_{16}H_{13}N_{10}O_{5}: C, 64.21; H, 4.38; N, 4.68. Found: C, 64.17; H, 4.33; N, 4.64.

β-Benzoyl-glycine ethyl ester. — To a solution of 2.94 g (21 mmoles) of glycine ethyl ester hydrochloride with 21 mmoles of triethylamine in 100 ml of anhydrous chloroform, 5.98 g (20 mmoles) of β-benzoyl-propionyl-p-nitrophenyl ester were added, and the mixture was stirred for 48 hrs. After removing the precipitate of the triethylammonium salt and alkaline and acidic washings, the solvent was removed in vacuo and the residue crystallized from methanol-ethyl ether. The yield was 3.94 g (35%), mp 78°.

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washed with 3 l of 1 N HCl releasing, after concentra-
tion, 1.5 g of z-aminobenzoyl-propionic acid hydrochlor-
ide chromatographically and analytically identical to
the substance prepared from benzoyl acrylic acid. It
was then reduced with NaBH\(_4\) to \(\gamma\)-phenyl-homoserine
which also gave two distinct peaks when chromato-
graphed in the amino acid analyzer. This fact suggested
that the two peaks had to be related to the existence
of the two pairs of enantiomers.

\textit{Carbobenzoxy-\(z\)-amino-\(\beta\)-benzoyl-propionic acid.} A
solution of 1.93 g (10 mmoles) of \(z\)-amino-\(\beta\)-benzoyl-
propionic acid in 200 ml of 1 M NaHCO\(_3\), cooled at
0\(^\circ\), was added under constant stirring of 2.04 g (12
mmoles) of benzyloxy carbonyl chloride, the pH of
the solution being continuously held at pH 9 by addition
of NaOH. After 3 hrs the solution was extracted with
ether, cooled at 0\(^\circ\) and acidified to Congo red
with 5 N HCl. The precipitate, formed on standing,
was collected, washed with water and dried. The yield
was 2.84 g (87\%), mp 128\(^\circ\); ninhydrin negative spot
\((R_1 \, 0.65; \, R_2 \, 0.35).$

An al. Calcd. for C\(_{24}\)H\(_{28}\)NO\(_7\): C, 66.05; H, 5.24;
N, 4.28. Found: C, 66.15; H, 5.25; N, 4.15.

\textit{Carbobenzoxy-\(z\)-amino-\(\beta\)-benzoyl-propionyl-p-nitrope-
nyl ester.} \textit{—} To a solution of 3.27 g (10 mmoles) of
\textit{carbobenzoxy-\(z\)-amino-\(\beta\)-benzoyl-propionic acid, 1.5 g
(11 mmoles) of p-nitrophenol in 60 ml of anhydrous chloroform cooled at 0\(^\circ\), 2.06 g (12 mmoles) of
dicyclohexyl carbodiimide were added under stirring.
After 48 hrs at room temperature the solution was
washed with 5\% NaHCO\(_3\), dried over Na\(_2\)SO\(_4\), con-
centrated in vacuo. The residue, crystallized from
ethyl acetate-ethyl ether gave yellow crystals (4.12 g) melting
at 224\(^\circ\), and giving a single spot, hypochlorite starch
positive, \(R_1 \, 0.9).$

\textit{An al. Calcd. for C\(_{24}\)H\(_{29}\)N\(_2\)O\(_7\): C, 64.28; H, 4.50;
N, 6.25. Found: C, 64.00; H, 4.47; N, 6.28.}$

\textit{Carbobenzoxy-\(z\)-amino-\(\beta\)-benzoyl-propionyl-glycine}
e\textit{ethyl ester.} \textit{—} To a solution of 1.7 g (12 mmoles) of
glycine ethyl ester hydrochloride and 1.5 g of oxalic acid in 20 ml of 50\%
ethanol, 1.5 g of \(\text{NaBH}_4\) were slowly added under stirring. After 48 hrs at room temperature the
solution was concentrated to dryness in vacuo. The
ethyl acetate solution of the residue was washed with
1 N HCl, 5\% NaHCO\(_3\) solution and water. After drying
over Na\(_2\)SO\(_4\) the solution was concentrated in vacuo
and added of petroleum ether. After standing for
several hours at 0\(^\circ\), a crystalline product separated out.
The yield was 2 g (83\%), mp 135\(^\circ\).

\textit{An al. Calcd. for C\(_{24}\)H\(_{29}\)N\(_2\)O\(_7\): C, 62.10; H, 6.05;
N, 8.69. Found: C, 62.40; H, 6.02; N, 8.83.}$

\textit{Carbobenzoxy-\(z\)-amino-\(\beta\)-benzoyl-propionyl-p-nitrope-
nyl ester.} \textit{—} To a solution of 2.42 g (5 mmoles) of carbo-
benzoxy-\(z\)-amino-\(\beta\)-benzoyl-propionyl-glycine ethyl
ester and 1.5 g of oxalic acid in 20 ml of 50\%
ethanol, 1.85 g (50 mmoles) of \(\text{NaBH}_4\) were slowly
added under stirring. At the end of the reaction,
checked with the disappearance of reactivity toward
2,4-dinitrophenyl-hydrazine, few drops of conc. HCl
were added to destroy the excess of \(\text{NaBH}_4\). The reaction
mixture was diluted with water, freed from MeOH
by vaporization under reduced pressure and extracted with
ethyl acetate. After washing with water, solution
was evaporated to dryness and the residue, crystallized
from ethyl acetate-ethyl ether. The yield was 1.37 g, mp 148\(^\circ\).

\textit{An al. Calcd. for C\(_{24}\)H\(_{29}\)N\(_2\)O\(_7\): C, 61.84; H, 6.44;
N, 8.66. Found: C, 61.76; H, 6.38; N, 8.60.}$

\textit{Alanyl-\(\gamma\)-phenyl-homoseryl-glycine ethyl ester.} \textit{—} To a solution of carbobenzoxy-alanyl-\(\gamma\)-phenyl-homoseryl-glycine
ester (1 g) in 100 ml of methanol, was hydrogenated in the presence of 10\% palladium on
charcoal. After 12 hrs the catalyst was removed and
the solution was added of equimolecular amount of
dry HCl in methanol and concentrated to dryness. The
residue was crystallized from methanol-ethyl ether. The
yield was 0.59 g, mp 138\(^\circ\); single ninhydrin positive
spot \((R_1 \, 0.68; \, R_2 \, 0.40).$

\textit{An al. Calcd. for C\(_{14}\)H\(_{16}\)N\(_2\)O\(_4\): HCl: C, 52.71; H,
6.72; N, 10.85. Found: C, 52.68; H, 6.58; N, 10.79.}$

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