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Ammonolysis of Peptides: A Method of Determining C-Terminal Amino Acids[1,2]

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The products formed in the ammonolysis of various peptides in liquid ammonia have been investigated. A procedure was devised by which the ammonolysis reaction was used as the basis of a method to detect the C-terminal amino acid in a peptide chain. This method was tested on twenty-five peptides representing sixteen different C-terminal amino acids. From the results so obtained, the potentialities and limitations of this method for the determination of C-terminal amino acids in unknown peptides and proteins have been assessed.

Although the hydrolytic cleavage of the peptide bonds in peptides and proteins has been extensively studied over a number of years, very little has been reported on the degradation of these compounds in non-aqueous solutions, such as liquid ammonia. In 1931, McChesney and Miller[4] treated several proteins with liquid ammonia in the presence of various ammono acids (such as ammonium chloride) and ammono bases (such as potassium amide). Although they obtained water soluble materials from their reactions, they were unable to characterize the products with the techniques available to them at that time.

After the ammonolytic cleavage of the peptide bonds in a protein, one would expect to obtain amino acid amides from each amino acid residue whose carboxyl group participates in a peptide bond. Amino acid residues which occur at the carboxyl end of a peptide chain (C-terminal amino acid) should be liberated as a free amino acid. The present report deals primarily with investigations performed in testing this reaction as a method for the detection of the C-terminal group in peptides.

Akabori, Ohno, *et al.*,[5] have proposed a similar reaction involving hydrazinolysis for the detection of C-terminal amino acids in peptide chains and have described their results with a number of proteins. The findings by the hydrazinolysis method have not always been in agreement with the reports of other authors. For example, by this method alanine and glycine were detected as the C-terminal amino acids in crystalline beef insulin.[5a] At the time of this report the findings were in agreement with the results of Fromageot, *et al.*,[6] by the amino alcohol method. However, the carboxypeptidase method[7] and later studies by the amino alcohol method[8] indicate that alanine and asparagine are the C-terminal amino acids in crystalline beef insulin. In view of these conflicting reports it seemed advisable to study the ammonolysis reaction on a wide variety of known peptides, representing a large number of different C-terminal amino acids, before applying it to proteins.

Preliminary experiments were performed in order to determine the conditions necessary for complete ammonolysis of the peptide bonds in leucylglycine and glycylphenylalanine. In order to obtain appreciable ammonolysis of these peptides in a period of 48 hours, it was necessary to use a temperature in excess of 100°. The rate of ammonolysis was increased by the addition of ammonium ions. Thus, glycylphenylalanine was completely ammonolyzed in 48 hours at 100° in a solution which was 2 *N* in ammonium ions, while the reaction was incomplete in the absence of added ammonium ions. The conditions reported in the experimental part were somewhat in excess of this and as judged from the results obtained are deemed to be sufficiently vigorous to ensure complete ammonolysis of any normal peptide bond.

In order to apply the ammonolysis reaction to the determination of C-terminal amino acids, it was necessary to develop a method of detecting the free amino acids in the mixture of amino acid amides formed by the reaction. Although paper chromatography appeared to be the most expedient method of detecting the free amino acids, the use of this technique was complicated by the fact that the ammonium chloride introduced as a catalyst in the ammonolysis reaction caused severe streaking of the chromatograms. In addition, the production of a large number of ninhydrin-positive spots from the amino acid amides in the ammonolysate of a large peptide or protein made it difficult to identify the free amino acid on the chromatogram. In order to remove the salt and at the same time simplify the reaction mixture, the ammonolysate was passed through a strong base, ion-exchange resin. The ammonium ions and the amino acid amides, except those with an acidic function, such as tyrosinamide and the monoamides of aspartic and glutamic acids, passed through the resin and were collected in a fraction called the *amide fraction.* The free amino acids, with the exception of arginine, were retained on the resin and were subsequently eluted with acid to give the *acid fraction.* A flow sheet of the reaction and separation procedure is shown in Fig. 1.

Although it would only have been necessary to investigate the acid fraction in order to identify the C-terminal amino acid, it was decided to study the amide fraction as well and thus obtain further information on the over-all reaction products of

(1) For a preliminary report of this investigation see F. H. Carpenter and R. W. Chambers, *Federation Proc.*, **13**, 189 (1954).

(2) This work was supported in part by grants from Eli Lilly and Company and from the National Institute of Arthritis and Metabolic Diseases.

(3) Part of this investigation was performed during the tenure of a du Pont Postgraduate Fellowship in Biochemistry.

(4) E. W. McChesney and C. O. Miller, THIS JOURNAL, **53**, 3888 (1931).

(5) (a) S. Akabori, K. Ohno and K. Narita, *Bull. Chem. Soc. Japan*, **22**, 214 (1952); (b) S. Akabori and K. Ohno, *II^e Congrès international de biochimie* (Paris, Juliet 1952), Resumés des communications, p. 47; K. Ohno, *J. Biol. Chem. Japan*, **40**, 621 (1953); S. Akabori, K. Ohno, T. Ikenaka, A. Nagata and I. Haruna, *Proc. Japan Acad.*, **29**, 561 (1953).

(6) C. Fromageot, M. Jutisz, D. Meyer and L. Penasse, *Biochim. Biophys. Acta*, **6**, 283 (1950).

(7) J. I. Harris, THIS JOURNAL, **74**, 2944 (1952); F. Sanger and E. O. P. Thompson, *Biochem. J.*, **52**, iii (1952).

(8) P. Jolles and C. Fromageot, *Biochim. Biophys. Acta*, **9**, 416 (1952).

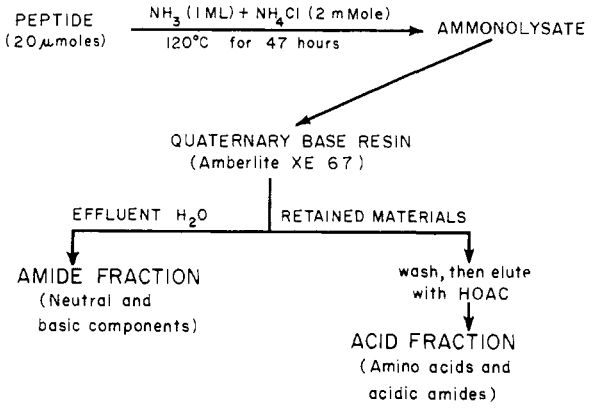


Fig. 1.—Flowsheet of the ammonolysis reaction and separation scheme.

ammonolysis. The examination of the amide fraction by paper chromatography meant that authentic samples of the amino acid amides had to be prepared as chromatographic standards. The preparation and characterization of these amino acid amides form the subject of a separate communication.[9]

Experimental

Procedure.—Twenty micromoles of peptide and 2 mmoles of dry ammonium chloride were placed in a micro-Carius combustion tube. Ammonia gas was condensed in the reaction tube under anhydrous conditions until 1 ml. of liquid ammonia had been collected and the tube was sealed. The tube was placed in a steel bomb which contained liquid ammonia (*ca.* 3 ml.) to equalize the pressure on the glass tube. The lower section of this bomb was machined from 1 inch, hexagonal stock and the cap from 1.5 inch, round stock, cold rolled steel. The threads were V-type, eight threads to the inch. A Teflon gasket was fitted into the top to give a tight seal.

After the bomb had been heated in an oil-bath at 120 ± 2° for 47 hours, it was cooled in Dry Ice for about ½ hour before opening. The glass reaction tube was removed and the steel bomb could then be used for a new reaction without replacing the ammonia it contained.

The glass reaction tube was cooled in a Dry Ice-bath, opened and two alundum boiling chips were added. If any solid cake was present it was broken up with a glass rod and the ammonia was allowed to evaporate at room temperature. After the last traces of ammonia had been removed in a vacuum desiccator over sulfuric acid, the dry residue was dissolved in 1 ml. of carbon dioxide-free water and the solution passed through a small gravity filter onto an ion-exchange column prepared as described below.

The resin[10] was prepared. using carbonate-free reagents throughout, by removing the "fines" with several water washes and then cyclizing, three times, batchwise, with 5% sodium hydroxide and 1 *N* hydrochloric acid. The resin was stored in the chloride form. A column was prepared by pouring 2.6 ml. of dry resin (chloride form) into a glass column with an internal diameter of 8 mm. The resin was backwashed twice to settle the resin bed and then about 25 ml. of 5% sodium hydroxide was passed through the column at a flow rate of about 1 ml. per minute. The alkali was followed immediately by a 300 ml. of water wash at a flow rate of about 0.3 ml. per minute.

The reaction mixture was washed onto the column with two 1-ml. portions of water. The resin was then washed with 50 ml. of water at a flow rate of about 1 ml. per minute to elute the amino acid amides and the ammonium ions. The effluent constituted the *amide fraction*. The resin was washed with an additional 150 ml. of water and the effluent discarded. About 3 ml. of 1 *N* acetic acid was allowed to run through the column to bring the resin almost to the acid breakthrough point and the column was further developed with 25 ml. of 0.1 *N* acetic acid. The *p*H of the effluent was checked with *p*H test paper. In most experiments, some evidence of carbon dioxide formation was observed just before the acid breakthrough. This was accompanied by a rise in the effluent *p*H. When the *p*H returned to about 5, the collection of the *acid fraction* was started. The resin was then discarded.

The *amide fraction* and the *acid fraction* were concentrated separately to about 1 ml. under reduced pressure and transferred to a conical centrifuge tube where the volumes were reduced to about 50 λ under reduced pressure. A small drop of saturated thymol water was added to each fraction and a small drop of 1 *N* acetic acid was added to the amide fraction. Each fraction was then diluted with water to exactly 100 λ in a 100 λ pipet. The contents of the pipet were mixed by expelling them onto a spot plate which had been treated with an organosilicone.[11] The fractions obtained from peptides containing cysteine or cystine residues (glutathione and bis-leucyl-cystine) were diluted with 0.1 *N* N-ethylmaleimide[12] instead of water. The N-ethylmaleimide was added to cystine-containing peptides on the chance that some reduction may have occurred in the reaction.

The standard solution of known compounds used for chromatography contained 0.05 micromole of compound per 1 λ of solution. The standards were authentic samples of peptides (see Table I), amino acids and amino acid amides.[9] In certain cases, notably with prolylphenylalanine and tyrosine, it was necessary to add a drop of concentrated ammonium hydroxide in order to dissolve the solid completely.

Chromatography was carried out in jars by the descending method on strips (6 × 22 in.) of Whatman No. 1 filter paper. The solvent systems used were 1-butanol–acetic acid–water[13] and pyridine–isoamyl alcohol–water.[14] One lambda of the amino acid standards, 2 λ of the amide standards, 5 λ of the *acid fraction* and 5 of the *amide fraction* were applied in 1 λ portions to the paper. After chromatography, the papers were dried and then sprayed with a ninhydrin–collidine reagent[15] which was 1% in acetic acid. The spots were developed by heating the papers in an oven at 90° for 5 minutes. The colors and relative intensities of the spots, as compared with the standards, were noted and then the papers were sprayed with a copper chloride solution[15] (1% cupric chloride dihydrate in ethanol) to preserve the spots for photography.[16] 2-Pyrrolidone-5-carboxylic acid and 2-pyrrolidone-5-carboxamide were detected by the chlorine–starch–iodide method of Rydon and Smith.[17]

When the chromatograms of the amide or acid fraction showed a spot which did not correspond in R_f values, in both solvents, with the expected ammonolysis products of the peptide under investigation, the unknown spot was tentatively identified, where possible, by comparison with other authentic compounds. For further confirmation of this identification, the material was eluted from the paper and subjected to hydrolysis. The material in the hydrolysate was then compared chromatographically with authentic samples of the suspected hydrolysis products.

Results and Discussion

The results obtained after ammonolysis of 25 peptides and one protein are summarized in Table I. Consideration of the products found in the acid fractions indicates that 13 of the 16 different C-terminal amino acids represented in these peptides were detectable by the ammonolysis procedure. In almost every case, the only major component in the acid fraction was the expected C-terminal amino acid, although tyrosinamide was also found, as expected, after ammonolysis of peptides containing

(9) F. H. Carpenter and R. W. Chambers, THIS JOURNAL, **77**, 1522 (1955).

(10) Amberlite XE-67, Rohm and Haas Co., Resinous Products Division, Philadelphia, Pennsylvania.

(11) Desicote, Beckman Instruments, South Pasadena, California.

(12) C. S. Hanes, F. J. R. Hird and F. A. Isherwood, *Nature*, **166**, 288 (1950).

(13) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(14) P. Edman, *Arkiv. Kemi., Mineral. Geol.*, **22A**, 1 (1945).

(15) A. L. Levy and D. Chung, *Anal. Chem.*, **25**, 396 (1953).

(16) Photographs, which were made of all the significant chromatograms, are to be found in the doctoral dissertation of Robert Warner Chambers, University of California, Berkeley, 1954.

(17) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).

TABLE I

SUMMARY OF AMMONOLYSIS PRODUCTS

Peptide	Products: Acid fraction	Products: Amide fraction
Leu-Ala-Ala[a,b]	**Ala**	**Leu-NH_2**,[c] **Ala-NH_2**, *Ala*
Leu-Gly-Ala[b]	**Ala**, *Gly*	**Leu-NH_2**, Gly-NH_2, **Ala-NH_2**, *Gly*, *Unk*
Bis-Leu-Cys[b]	*Leu*, *Unk*	**Leu-NH_2**, Ala-NH_2, *Gly-NH_2*[d]
Arg-Glu[e]	**Py-CO_2H**, *Unk*	Py-$CONH_2$, Unk
Leu-Gly[b]	**Gly**, *Leu*	**Leu-NH_2**, Gly-NH_2
Ala-Gly-Gly[b]	**Gly**, *Ala*	**Ala-NH_2**, **Gly-NH_2**, Gly
GSH[f]	**Gly**, Py-CO_2H	**Ala-NH_2**, Gly-NH_2, **Py-$CONH_2$**
His-His[g]	**His**	**His-NH_2**, *Gly-NH_2*[d]
Gly-Leu[b]	**Leu**, *Gly*	**Gly-NH_2**, Leu-NH_2
Arg-Leu[e]	**Leu**, *Arg*	Leu-NH_2
Ala-Met[h]	**Met**, *Ala*, Unk	**Ala-NH_2**, Met-NH_2
Ala-Nval[h]	**Nval**, *Ala*	**Ala-NH_2**, Nval-NH_2
Gly-Phe[h]	**Phe**, *Gly*	**Gly-NH_2**, Phe-NH_2
Pro-Phe[b]	**Phe**, *Unk*	**Pro-NH_2**, Unk
Gly-Pro[h]	**Pro**, *Gly*	**Gly-NH_2**, Pro-NH_2
Gly-Try[h]	**Try**, *Gly*	**Gly-NH_2**, Try-NH_2
Gly-Tyr[g]	**Tyr**, **Tyr-NH_2**, *Gly*	**Gly-NH_2**
Gly-Val[g]	**Val**, *Gly*	**Gly-NH_2**, *Val-NH_2*
Ser	**Ser**, *Ala*	**Ala-NH_2**, Gly-NH_2
Ser-NH_2	*Ala*, *Gly*	**Ala-NH_2**, Gly-NH_2
Ser-Ser[b]	**Ser**, *Ala*	**Ala-NH_2**, Gly-NH_2
Gly-Ser[b]	**Ser**, *Ala*, *Gly*	**Ala-NH_2**, **Gly-NH_2**
Tyr-Ser[i]	**Ser**, **Tyr-NH_2**, Tyr, *Gly*	**Ala-NH_2**, Gly-NH_2
Ser-Gly[i]	**Gly**, *Ala*	**Ala-NH_2**, **Gly-NH_2**
Gly-Asp[b]	**Asp**, *Asp-NH_2*, *Iasp-NH_2*,[d] *Gly*	**Gly-NH_2**, Asp-DiNH_2, *Ala-NH_2*, *Asp-NH_2*
Leu-Asp[b]	**Asp**, *Asp-NH_2*, *Iasp-NH_2*[d]	**Leu-NH_2**, Asp-DiNH_2, *Ala-NH_2*, *Asp-NH_2*
Asp-NH_2	*Asp*, *Asp-NH_2*, *Iasp-NH_2*[d]	**Asp-DiNH_2**, *Asp-NH_2*
Leu-Asp-NH_2[b]	*Asp*, *Asp-NH_2*, *Leu*	**Leu-NH_2**, **Asp-DiNH_2**, *Asp-NH_2*
Iasp-NH_2	Iasp-NH_2, *Asp*, *Asp-NH_2*, *Gly*[d]	**Asp-DiNH_2**, *Asp-NH_2*, Ala-NH_2
Insulin[j]	**Ala**, *Asp-NH_2*, **Tyr-NH_2**, *Tyr*, *Gly*, *Unk*	Not investigated

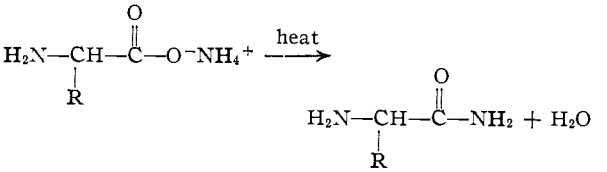
[a] The abbreviations used are those suggested by E. Brand and J. T. Edsall (*Ann. Rev. Biochem.*, **16**, 223 (1947)). In addition, the following abbreviations are used: Suffix -NH_2 = amino acid amide, *e.g.*, Ala-NH_2 = alaninamide; Iasp-NH_2 = isoasparagine; Asp-DiNH_2 = aspartic diamide; GSH = γ-glutamyl-cysteinylglycine (glutathione); Py-$CONH_2$ = 2-pyrrolidone-5-carboxamide; Py-CO_2H = 2-pyrrolidone-5-carboxylic acid; Unk = unidentified component. [b] Kindly supplied from the Emil Fischer Collection by Professor H. O. L. Fischer. [c] Compounds in bold face type represent the main components in the reaction mixture. Compounds in italics represent trace components which, on the basis of their spot density, were estimated to make up less than 10% of the reaction mixture. Compounds in regular type represent components which were not major constituents of the reaction mixture, but which were present in more than trace amounts. [d] The identity of this component is probably as indicated, but because of the small amount of material present, its identity could not be confirmed by the hydrolysis procedure. [e] Prepared in this Laboratory (D. T. Gish and F. H. Carpenter, THIS JOURNAL, **75**, 5872 (1953)). [f] Schwarz Laboratories, Inc., New York 17, N. Y. [g] Nutritional Biochemicals Corporation, Cleveland, Ohio. [h] Mann Research Laboratories, Inc., New York 6, N. Y. [i] Kindly supplied by Dr. J. I. Harris. [j] Crystalline Zn insulin, Eli Lilly Research Laboratories, Indianapolis, Ind.

a tyrosine residue (glycyltyrosine and tyrosylserine). Except in certain cases, which will be discussed later, the only additional products detected in the acid fraction, other than the C-terminal amino acid, were trace amounts of free amino acids, which were apparently produced by hydrolytic side reactions. Since the only water which could be present in the reaction mixture would be that formed by amidation and decomposition side reactions, it seemed unlikely that hydrolysis could successfully compete with the ammonolysis reaction in the presence of the large excess of ammonia. It is more likely that the hydrolytic side products were produced during the work-up procedure, either on the resin or during concentration of the effluents. To test this possibility, glycinamide and also glutamine were subjected to the separation scheme, without previous ammonolysis, and it was found that small amounts of the corresponding free amino acids were formed.

The amount of free amino acid formed from di- and tripeptides by these hydrolytic side reactions was relatively insignificant compared with the amount of free amino acid liberated from the C-terminal residue by ammonolysis. Consequently the hydrolytic products did not confuse the interpretation of the results with these simple peptides. However, with proteins, where the ratio of amides to free amino acids is very high, hydrolysis might become quite serious. Therefore, some modification of the separation scheme would seem to be necessary before ammonolysis can be applied routinely to the determination of the C-terminal residues in proteins.

Consideration of the amide fraction indicates that several side products were produced. With most of the 25 peptides investigated, the side reactions did not cause any ambiguities in the detection of the C-terminal amino acid, but with certain C-terminal residues in peptides (*e.g.*, glutamic, cystine and asparagine) and for application of this method to proteins, these side reactions might lead to uncertainties in the interpretation of the results.

Conversion of the the free carboxyl group of the C-terminal amino acid to its amide was the most common side reaction.

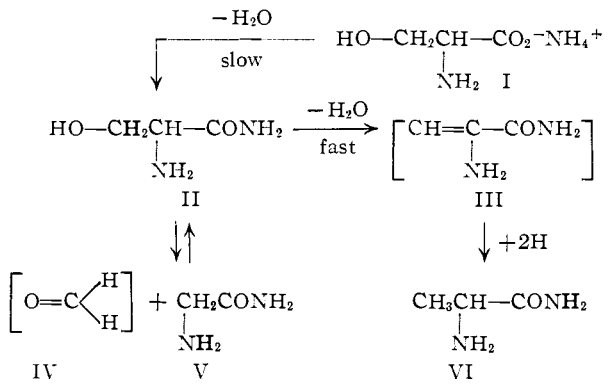


This reaction occurred with every peptide which was investigated, but the extent of the reaction varied with different C-terminal amino acids. For example, C-terminal glycine gave relatively large amounts of glycinamide, while C-terminal valine gave only small amounts of valinamide. The extent of this amidation reaction was also dependent upon temperature and catalyst concentration. An increase in either of these variables increased the

amount of amide formed. This side reaction does not preclude the detection of the C-terminal amino acid, but it decreases the sensitivity of the method and makes a quantitative estimation of the C-terminal residue difficult.

Glycine Derivatives.—Apparently, glycinamide underwent extensive decomposition to unknown products, since the reaction mixtures from peptides containing a glycyl residue (*e.g.*, glycylleucine) and to a lesser extent, peptides containing C-terminal glycine, were dark brown and contained material which was insoluble in water. This decomposition may explain why the amount of glycinamide detected in the ammonolysate of glycyl peptides was usually less than expected.

Serine Derivatives.—The products obtained by ammonolysis of serine, serinamide and several peptides containing serine residues are summarized in Table I. Although C-terminal serine was detectable, alaninamide and glycinamide were invariably produced as side products, even when free serine was subjected to ammonolysis. It should be pointed out that the amount of glycinamide which was detected appeared to be small compared with the amount of alaninamide found, except with those peptides which contained a glycine residue (glycylserine and serylglycine). However, the low yield of glycinamide may have been due to decomposition reactions (see above). The fact that no serinamide could be detected,[18] even when serinamide was subjected to ammonolysis, while C-terminal serine was always detectable, suggested that serinamide underwent decomposition more rapidly than free serine. Furthermore, it is important to notice that the end products of this decomposition reaction were always alaninamide and glycinamide rather than the corresponding free amino acids if one assumes that the trace amounts of alanine and glycine which were detected were produced by hydrolysis during the work-up procedure. This information suggested the following mechanism for the decomposition of these serine derivatives.



This series of reactions is similar to those postulated by Wieland and Wirth[19] to explain the formation of glycine and alanine by alkaline decomposition of serine in saturated barium hydroxide. Also, Nicolet, *et al.*,[20] postulated the formation of dehydroalanine as an intermediate in the partial alkaline decomposition of serine residues in proteins under conditions which did not measurably affect free serine. These results agree with our finding that serinamide (II) is rapidly decomposed to alaninamide (VI) whereas serine is not converted to alanine at an appreciable rate under the conditions of ammonolysis.

If dehydroalaninamide (III) is an intermediate in the formation of alaninamide, the mechanism of the reduction of III is obscure. This reduction may occur at the expense of the formaldehyde which is presumed to be formed in the decomposition of serinamide to glycine.

One feature of this mechanism is that a similar series of reactions can be used to explain the production of alaninamide from peptides containing a cysteine residue (glutathione) or a cystine residue (bis-leucylcystine). For example, the loss of hydrogen sulfide from cysteinamide by β-elimination to give dehydroalaninamide, and eventually alaninamide, is exactly analogous to the elimination of water from serinamide. The fact that we could not detect any cysteinamide among the ammonolysis products from glutathione, plus the fact that the odor of hydrogen sulfide was quite marked after removal of the ammonia from the reaction mixture, supports this mechanism.

Glutamic Acid Derivatives.—Another C-terminal amino acid which could not be determined by the ammonolysis procedure was glutamic acid. With arginylglutamic acid (see Table I), the main product detected was 2-pyrrolidone-5-carboxylic acid. Small amounts of 2-pyrrolidone-5-carboxamide, as well as two other unidentified components were also detected. A similar side reaction occurred with glutathione, except that the major product from the γ-glutamyl residue was 2-pyrrolidone-5-carboxamide rather than the free acid. It is also interesting to note that argininamide was not detected as an ammonolysis product of either arginylleucine or arginylglutamic acid.

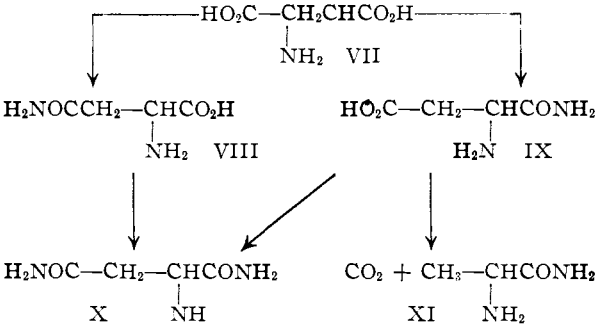
Aspartic Acid Derivatives.—Consideration of the results obtained with aspartic acid derivatives (see Table I) shows that it was possible to detect C-terminal aspartic acid, but not C-terminal asparagine. It is also important to notice that aspartic diamide was a product with every one of the aspartic acid derivatives which was studied. Furthermore, alaninamide was detected in small amounts after ammonolysis of isoasparagine, leucylaspartic acid and glycylaspartic acid. However, no alaninamide was detected when asparagine was the C-terminal amino acid. When asparagine was subjected to ammonolysis, the main product which was detected was aspartic diamide. These results may be summarized by the series of reactions shown beyond.

This reaction scheme may be explained by competitive formation of the α- or β-amide. If the α-amide (X, isoasparagine) formed first, then decarboxylation to alaninamide (XI) as well as amidation to aspartic diamide (IX) occurred. If, on the

(18) It was necessary to run two-dimensional chromatograms in butanol–acetic acid–water followed by pyridine–isoamyl alcohol–water, in order to rule out serinamide, since it ran with glycinamide in butanol–acetic acid and with alaninamide in pyridine–isoamyl alcohol.

(19) T. Wieland and L. Wirth, *Ber.*, **82**, 468 (1949).

(20) B. H. Nicolet, L. A. Shinn and L. J. Saidel, *J. Biol. Chem.*, **142**, 609 (1942).



other hand, the β-amide (VIII, asparagine) formed first, then decarboxylation did not occur and VIII was further amidated to IX.

When crystalline beef insulin was subjected to ammonolysis, the only C-terminal acid which could be detected, with certainty, was alanine, although traces of asparagine and glycine were also found (see Table I). The failure to find more than a trace of asparagine was attributed to the conversion of asparagine to aspartic diamide.

Conclusions.—Table II summarizes the applicability of the ammonolysis procedure to the determination of the C-terminal amino acids in peptides. Of the 16 different C-terminal amino acids studied, only 3, asparagine, cystine and glutamic acid, could not be detected in peptides by this procedure.

The main disadvantage of the present ammonolysis procedure is that it cannot be applied routinely to proteins. However, modification of the separation scheme may obviate this difficulty. It should be noted that the ammonolysis procedure has been investigated more extensively with known compounds than any other chemical method of determining C-terminal amino acids. The irregularities encountered in the experiments reported here demonstrate the dangers inherent in drawing conclusions from any C-terminal method until it has been tested on a wide variety of known compounds in which most of the possible C-terminal amino acids are represented. This is particularly significant in view of the conflicting reports in the literature concerning the identity of the C-terminal amino acids in certain proteins, such as insulin. It should also be noted that some of the side reactions encountered in the ammonolysis reaction might be expected to occur in the hydrazinolysis procedure of Akabori.[5] In fact Locker has just recently reported[21] that arginine, cysteine, cystine, aspartic acid, asparagine, glutamic acid and glutamine are destroyed on heating in anhydrous hydrazine at 108° for 10 hours. These are the same amino acids which we judged to be non-detectable in the ammonolysis procedure described here.

TABLE II

DETECTABILITY OF THE C-TERMINAL AMINO ACIDS IN PEPTIDES BY THE AMMONOLYSIS PROCEDURE

Investigated		Not investigated		
Detectable		Not detectable	Probably detectable	Probably not detectable
Alanine	Norvaline	Asparagine	Isoleucine	Cysteine
Aspartic acid	Serine	Cystine	Lysine	Glutamine
Glycine	Methionine	Glutamic acid	Threonine	Isoasparagine
Histidine	Tryptophan			Isoglutamine
Leucine	Tyrosine			Arginine
Phenylalanine	Valine			
Proline				

(21) R. H. Locker, *Biochim. Biophys. Acta*, **14**, 533 (1954).

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[CONTRIBUTION FROM THE DEPARTMENTS OF PHARMACOLOGY, UNIVERSITY OF TENNESSEE, AND BIOCHEMISTRY, UNIVERSITY COLORADO MEDICAL SCHOOL AND OAK RIDGE NATIONAL LABORATORY]

Enzyme Inhibition by Complexing of Substrates: Inhibition of Tyrosinase by Titanium Compounds

BY D. J. CAVANAUGH, JOSEPH HARRIS[1] AND J. Z. HEARON

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The oxidation of O-dihydric phenolic substrates by tyrosinase was found to be inhibited by Ti^{IV} and triethanolamine titanate. The kinetics of this inhibition were in accord with the hypothesis that it was due to competition between enzyme and inhibitor for the substrate rather than between substrate and inhibitor for the enzyme. The details of the kinetics with regard to 3,4-dihydroxyphenylalanine as the substrate have been presented here. This type of inhibition clearly demonstrated one of the possible modes of action in enzyme systems of metals capable of forming complexes with the substrates.

The effects of metal–substrate reactions[2,3] and of complex ions[4] on enzymatic reactions have been examined to some extent. The case of a type of competitive inhibition arising from competition between enzyme and metal or metal–complex for the substrate has not received detailed consideration. This paper is concerned with such inhibition as it occurs between tyrosinase and the complex, triethanolamine titanate (TAT).[5]

The formation and analytical employment of Ti complexes, *e.g.*, with 1,2-dihydroxybenzene-3,5-disulfonate, have been studied.[6–9] The structural

(1) A portion of the preliminary work was done by J. H. at Baxter Laboratories, Inc., Morton Grove, Illinois.

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