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Terminal amino acid residues appearing during the proteolysis of lysozyme

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NOTE TO USERS

TERMINAL AMINO ACID RESIDUES APPEARING DURING THE PROTEOLYSIS OF LYSOZYME

by

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

INTRODUCTION

By determining the terminal amino acid residues present at various times during the proteolysis of lysozyme information was sought regarding:

1. The course of the proteolysis of lysozyme.
2. The preference of the enzymes under study for the peptide linkages in lysozyme.
3. Some of the amino acid sequences in lysozyme.
4. The applicability of the quantitative aminoid sequence method (41) to proteolytic studies.

In order to determine the amino acid residues at the carboxoid termini of the peptide chains, work was undertaken to develop a quantitative carboxoid residue assay to be used in conjunction with the quantitative aminoid residue method previously developed (55).

The pioneering work of Northrop, Kunitz, and Herriot (75) and of Anson (5) on the purification and crystallization of enzymes made possible a more critical experimental approach to the action and specificities of proteolytic enzymes.

Bergmann and coworkers (12, 13, 14, 16) have shown that for some of these enzymes synthetic peptides and peptide derivatives can take the place of the more complex

protein substrates for studying the specificities of these purified enzymes.

The extensive work of Bergmann and the numerous advantages of using synthetic substrates for these investigations relaxed attention from the use of natural substrates for several years. Interest in the use of natural substrates is now reviving mainly because of the development of new techniques such as chromatography and quantitative terminal residue assay methods.

REVIEW OF LITERATURE

Proteolytic Studies

Extensive reviews and appraisals of methods for following proteolysis have been made by Martin and Synge (72) and by Neurath and Schwert (74). Most of the methods are either applicable only to simple synthetic peptides or derivatives and are not specific for the individual amino acid residues. The alcohol titration, formol titration, and acetone titration are the simplest and have been most widely used, but they are specific only in the sense that the first two methods determine free amino groups and the titration in acetone determines free carboxyl groups. Practical difficulties also interfere with their usefulness. The gasometric nitrous acid method is time consuming, requires a relatively large aliquot for analysis, and is specific only for free amino groups. The gasometric ninhydrin procedure requires the presence of a free amino acid, so it has a limited utility, as is also true for the titrimetric ninhydrin method. The colorimetric ninhydrin method can be applied on a micro scale to follow proteolysis, but it still requires free amino acids or close standardization for simple peptides, so that its usefulness resides in the use of

simple peptides or derivatives as substrates.

Some of these methods, when coupled with chromatographic, electrophoretic, or other types of separation, can give interesting information regarding the course of the proteolysis, as demonstrated by Ingram (56). He examined the self-digestion of pepsin and found it to be rapid at 45° C. and pH 4. After 24 hours, 40 per cent of the total tyrosine of the protein precipitated out. Chromatographic techniques showed that a large number of peptide fragments were formed even at the start. Three peptides of considerable size were isolated from the chromatograms. Treatment of these peptides with nitrous acid, subsequent hydrolysis, and chromatography led to the conclusion that each of these peptides originally contained but one alanine residue which was always the amino bearing end group.

Synge (92) has reviewed the work of earlier investigators in their attempts to fathom protein structure by studying products of partial hydrolysis.

Butler *et al.* (28) studied the action of chymotrypsin and trypsin on insulin. At intervals, they determined non-protein nitrogen, Van Slyke amino nitrogen, total nitrogen, and free amino acids by the ninhydrin procedure. They concluded that crystalline trypsin has no appreciable effect on insulin but is capable of digesting the products formed after chymotryptic action. Also, the first stage of chymo-

tryptic action leads to the rapid formation of 50 per cent non-protein nitrogen. The second stage consists of a slow rise of non-protein nitrogen to the 100 per cent value. Insulin's physiological properties disappear at about the end of the first stage. Diffusion experiments showed that the "protein-like" material remaining at the end of the first stage of the chymotryptic hydrolysis had a molecular weight of about 4000, while that of the soluble fraction was about 800. The initial action of chymotrypsin thus involves the appearance of ten or eleven peptides containing an average of five or six residues. The amino nitrogen of the "protein core" indicates a probable chain length of 10-12 residues. Most of the cystine is contained in it. The number of bonds in insulin split by chymotrypsin is approximately equal to the number of aromatic amino acid residues contained in it as shown by Butler et al. (29). From the chromatographic examination of peptide fractions, it was concluded that chymotrypsin attacks preferentially bonds involving the carboxyl side of an aromatic residue.

Desnuelle, Rovery, and Bonjour (36) investigated the action of pepsin on horse globin and albumin, and Rovery, Desnuelle, and Bonjour (78) investigated the tryptic and chymotryptic hydrolysis of horse globin. It was found that each enzyme acts differently on globin and that the action of pepsin on globin is quite different from that on albumin.

The fragments of proteolysis were separated into high and low molecular weight fractions by means of trichloroacetic acid. Aminoid terminal serine and threonine were determined in these fractions by oxidation with periodate. Other aminoid terminal amino acid residues were determined by the reaction with 2,4-dinitrofluorbenzene and chromatographic separations as demonstrated by Sanger (79). The terminal amino acids on the other side of the split peptide bonds possessed free carboxyl groups, but they were not characterized.

It was found that pepsin at first split horse globin into very large peptides and medium sized peptides of about twelve residues. The large peptides were then reduced to about the size of the latter. Peptide bonds involving the aminoid groups of alanine, phenylalanine, leucine, and serine were at first preferentially hydrolyzed. On further hydrolysis, the dodecapeptides were reduced to tri- or tetrapeptides with no apparent selectivity. Albumin was broken down into penta- or hexapeptides with no preference for linkages demonstrated.

Trypsin and chymotrypsin were unable to hydrolyze horse globin to the point of complete solubility in trichloroacetic acid solution, but it was shown that trypsin acted preferentially on linkages involving the aminoid groups of alanine, phenylalanine, and threonine and chymotrypsin on

alanine, phenylalanine, threonine, and serine.

Specific differences in the action of pepsin and trypsin on bovine plasma albumin were shown by Riesen and Elvehjem (77) by their studies of the nutritional values of peptides produced for certain bacteria. Pepsin liberated larger quantities of peptides than did trypsin. Also, pepsin made available to the organisms larger quantities of all the amino acids except the basic ones and tryptophan. The latter were released in more utilizable form by trypsin.

Blackburn (24) partially characterized peptides which were formed on the digestion of wool by papain. In the water-soluble portion of the digest, considerable glycine, small amounts of serine and threonine, and peptides of about five residues of the basic amino acids were liberated.

Sizer (88) investigated the action of pepsin, trypsin, and chymotrypsin on collagen. Collagen was rapidly digested by pepsin in acid solution. It was relatively resistant to trypsin and chymotrypsin, but the rate was remarkably increased when the tendon was cut into very short lengths.

Using a commercial pepsin preparation, Moring-Claesson (73) examined the effect on crystalline egg albumin. Only part of the protein molecules were attacked at first and intact protein was observed until the very end of the reaction. The cleavage products at the beginning consisted of deca- or higher peptides, but, at the end, tripeptides were the

average constituents, and some dipeptides were indicated. The peptide fractions were investigated by paper chromatography and found to contain, mainly, glutamic acid, aspartic acid, glycine, valine, alanine, and leucine.

By means of electrophoretic separations, Boulanger, Biserte, and Swyngedauw (26) investigated the action of pepsin on egg albumin and serum albumin under various conditions. Proteolysis in M/10 phosphoric acid for 48 hours, followed by electrophoresis in phosphate buffer at pH 5.9, showed the presence of four basic components, four acidic components, and one neutral component.

Beloff and Anfinsen (11) studied the products of proteolytic digestion of egg and serum albumins, gamma globulin, and fibrin. Dialysates or filtrates from trichloroacetic or picric acid precipitations of the digests were assayed for free amino nitrogen by the Van Slyke nitrous acid method (96) and free amino acid nitrogen by the ninhydrin method (97). The amino acids bound in peptides were determined by the ninhydrin method after complete hydrolysis in acid. The ratio of the terminal amino nitrogen and the total nitrogen indicated the number of amino acid residues per peptide. During peptic, as well as tryptic, digestion, this ratio was close to three and was constant throughout the period of digestion. Peptic digestion of fibrin showed comparatively large amounts of free amino acids liberated in the first 30 minutes.

Earlier, Winnick (100) had employed a similar approach, but slightly different technique, in studying protease action on casein. Using pepsin, trypsin, chymotrypsin, papain, ficin, and carboxypeptidase, he found the average non-protein molecules contained from five to seven amino acid residues, with 1.5 to 4.5 per cent of the total nitrogen in the form of free amino acids.

A simple method for following the progress of protease action was described by Kunitz (67). The method consists of precipitating the protein still remaining by means of a 5 per cent trichloroacetic acid solution, centrifuging, decanting or filtering, and reading the optical density of the clear solution in a spectrophotometer at 280 millimicrons. The absorption at this wave length is due to the aromatic amino acids in solution, either as free amino acids or in combination in soluble peptides.

Fraser and Powell (42) utilized Kunitz's method in their investigation of the kinetics of trypsin self-digestion and tryptic digestion of purified casein.

Sanger and Tuppy (81, 82) used partial hydrolysis of the phenylalanine chain (the B chain) of insulin by pepsin, trypsin, and chymotrypsin in elucidating the amino acid sequence. By applying the dinitrofluorobenzene technique and identifying terminal aminoid residues, together with the sequence as worked out, they were able to postulate the

points of attack by the three enzymes. Pepsin appears to attack rather strongly the leucyl-valyl bond, the tyrosyl-leucyl bond, and the two linkages of phenylalanyl-phenyl-alanyl-tyrosyl. To a lesser extent phenylalanyl-valyl bond, glutamyl-histidyl, glycyl-phenylalanyl, and the three bonds in glutamyl-alanyl-leucyl-tyrosyl are split.

Chymotrypsin to the largest degree split the tyrosyl-leucyl bond and the bonds of phenylalanyl-tyrosyl-threonyl and to a lesser degree split the leucyl-tyrosyl bond.

Trypsin split the arginyl-glycyl and the lysyl-alanine linkages.

Recently Sanger and Thompson (80) employed peptic and chymotryptic digestion of the glycyl chain (fraction A) of insulin in determining its amino acid sequence. By using the techniques of paper chromatography for separating the peptide fractions, followed by reaction with 2,4-dinitro-fluorobenzene with subsequent partial hydrolysis and chro-matographic determinations, they were able to arrive at a sequence for the twenty-one amino acid residues in this chain. By the dinitrofluorobenzene method, they determined the amino acid residues whose amino groups were freed by the protease (cf. 37), and on reconstructing the molecule they could postulate, in a somewhat quantitative manner, which linkages were attacked by the protease. Trypsin was found not to hydrolyze the A chain (cf. 28). Pepsin

cleaved the leucyl-tyrosyl bond and the bonds of leucyl-glutamyl-aspartyl, but not aspartyl-tyrosyl linkage. Other bonds were hydrolyzed to a small extent, the glutamyl-glutamyl bond, the valyl-cysteic acid bond, the tyrosyl-glutamyl bond, and the glutamyl-leucyl bond. Chymotrypsin hydrolyzed the tyrosyl-glutamyl bond (cf. 29) and to a lesser extent the tyrosyl-cysteic acid bond and the cysteic acid-serine bond.

The preference of trypsin for the carboxoid groupings of arginine and lysine agrees with the specificities of this enzyme from synthetic peptide studies. The action of chymotrypsin, again to a certain extent, typifies the specificities as found with synthetic substrates in which the carboxoid end of the aromatic amino acids is preferentially hydrolyzed. Pepsin has been shown to hydrolyze both the aminoid and carboxoid termini of the aromatic amino acids in synthetic substrates, but the action on other linkages has not been demonstrated with peptides.

Currie and Bull (32) showed that at pH 3.5 and higher the action of pepsin on egg albumin itself is negligible. Pepsin tends to act as a proteinase at low pH values and as a peptidase at higher pH values. Bull and Currie (27) showed that the rate of proteolysis of crystalline egg albumin by pepsin was greatest at a pH value of 2 or less.

Behrens and Bergmann (10) have pointed out the possi-

bility of simultaneous synthesis and hydrolysis, so that sequences which did not exist in the original protein might be found after proteolysis. They showed that cysteine-papain would not hydrolyze acetyl-DL-phenylalanylglycine, glycinanilide, glycinamide, or glycyl-L-leucine. However, when acetyl-DL-phenylalanylglycine was present with any of the other three compounds, cleavage occurred. It was shown that acetyl-DL-phenylalanylglycine combined with glycyl-L-leucine to form acetyl-DL-phenylalanylglycylglycyl-L-leucine. This was hydrolyzed to produce first L-leucine and then glycine, leaving acetyl-DL-phenylalanylglycine intact.

Waley and Watson (99) found that, in the degradation of certain tripeptides by a mixture of trypsin and chymotrypsin, the products include dipeptides in which the sequence of amino acids residues differs from that in the original tripeptide. Lysyllysine could be obtained from lysyltryosyllysine by a mechanism in which the hexapeptide lysyltyrosyllysyllysyltyrosyllysine is formed as an intermediate. This then breaks down into lysyltyrosine, lysyllysine, and tyrosyllysine. Other workers have shown analogous reactions to take place (15, 46, 57).

These facts must be kept in mind when explaining or interpreting protease action.

Specificity of Enzymes

The term "specificity", which is so often applied to describing the actions of enzymes on limited types of substrates, at least with the proteases as knowledge is gained, is being supplanted by the word "preference". This word, in an approximate manner, is descriptive for relative rates of hydrolysis of various substrates.

Bergmann and Fruton (16), Neurath and Schwert (74), Fruton (49), and Balls and Jansen (7) have summarized protease action on synthetic peptides. These peptides and derivatives are given below, with the slant indicating the point of attack by the particular enzyme:

Specificity of trypsin

Acylarginine/amides (19, 20, 54)

Acylarginine/esters (86, 87)

Benzoylglycyl-L-lysine/amide (54)

Acyllysine/amides (53)

Specificity of chymotrypsin

Acyltyrosine/amides (48, 60, 62)

Benzoyltyrosine/ethyl ester (62)

Benzoyltyrosyl/glycinamide (18)

Benzoylphenylalanine/esters (60, 89)

Benzoylphenylalanine/amide (74)

Benzoylarginine/methyl ester (87)

Specificity of chymotrypsin (continued)

Benzoylmethionine/ethyl ester (60)

Benzoylnorleucine/ethyl ester (74)

Benzoylnorvaline/ethyl ester (74)

Acetyltyrosyl/glycinamide (74)

Acetylphenylalanine/amide (74)

Acetyltryptophan/ethyl ester (60)

Nicotinylmethionine/amide (74)

Nicotinyltryptophan/amide (74)

Glycyltyrosine/amide (48)

Glycyltyrosyl/glycinamide (17)

Glycylphenylalanine/amide (48)

Glycylglycine/amide (48)

Carbobenzoxytyrosyl/glycinamide (17)

Carbobenzoxytyrosyl/glycylglycinamide (17)

Carbobenzoxyphenylalanyl/glycinamide (48)

Carbobenzoxyglycyltyrosine/amide (48)

Carbobenzoxyglycyltyrosyl/glycinamide (17)

Carbobenzoxyglycylphenylalanyl/glycinamide (17)

Carbobenzoxyglutamyltyrosyl/glycinamide (16)

Glutamyltyrosyl/glycinamide (16)

Phenylalanyl/glycinamide (48)

Tyrosine/amide (48)

Tyrosyl/glycinamide (48)

Tyrosine/ethyl ester (60)

Bonds resistant to chymotrypsin

D-Leucylglycylglycine (68)

Chloracetyl-L-tyrosine (17)

Benzoyl-L-leucyl-L-leucylglycine (17)

Carbobenzoxyglycyl-L-leucylglycinamide (17)

Carbobenzoxyglycyl-L-glutamylglycinamide (17)

Benzoyl-DL-serine ethyl ester (62)

Benzoyl-DL-threonine ethyl ester (62)

Acetyl-DL-threonine ethyl ester (62)

Nicotinyl-DL-histidinamide (74)

Benzoylalaninamide (18)

Carbobenzoxy-L-tyrosylglycine (17)

Benzoylglycyl-L-lysineamide (17)

Specificity of pepsin

Carbobenzoxy-L-glutamyl-/L-tyrosine (47)

Carbobenzoxy-L-glutamyl-/L-phenylalanine (47)

Carbobenzoxy-L-glutamyl-/L-tyrosylglycine (47)

Carbobenzoxyglycyl-L-glutamyl-/L-tyrosine (47)

Glycyl-L-glutamyl-/L-tyrosine (47)

Carbobenzoxy-L-tyrosyl-/L-cysteine (51)

Carbobenzoxy-L-tyrosyl-/L-cystine (51)

Carbobenzoxy-L-cysteyl-/L-tyrosine (51)

Carbobenzoxy-L-cystyl-/L-tyrosine (51)

L-Tyrosyl-/L-cysteine (51)

L-Cysteyl-/L-tyrosine (51)

Specificity of pepsin (continued)

L-Cystyl-/L-tyrosine (51)

Carbobenzoxy-L-methionyl-/L-tyrosine (35)

L-Methionyl-/L-tyrosine (35)

Acetyl-L-tyrosyl-/L-tyrosine (6)

Acetyl-L-phenylalanyl-/L-phenylalanine (6)

Carbobenzoxy-L-tyrosyl-/L-phenylalanine (6)

Bergmann and co-workers showed that the preference of pepsin for a peptide bond depends not only upon the nature of both amino acid residues conjoined, but also upon the sequence of the amino acid residues.

Specificity of papain-HCN

Benzoylglycyl/glycyl-L-leucylglycine (21)

Benzoylglycine/amide (21)

Benzoyl-L-arginine/amide (19)

Benzoylglycyl-/L-leucylglycine (21)

Benzoyl-L-lysine/amide (22)

Benzoyl-L-tyrosylglycine/amide (21)

L-Leucine/amide (10)

Benzoylglycyl-/L-histidinamide (23)

Benzoylglycyl-/L-lysylglycine (23)

Benzoylglycyl-/L-arginine amide (54)

Carbobenzoxyglycyl-/L-glutamylglycine (21)

Carbobenzoxyglycyl-/L-glutamylglycine ethyl ester (21)

Benzoylglycyl/glycyl-L-glutamylglycine ethyl ester (21)

Specificity of papain-HCN (continued)

Carbobenzoyglycyl-/L-asparagylglycine ethyl ester (21)

Carbobenzoxy-L-glutamyl/glycine ethyl ester (21)

Carbobenzoxyglycyl/glycylglycine (21)

Carbobenzoxy-L-leucylglycyl/glycine (21)

Lysozyme

The discovery, purification, properties, and composition of lysozyme have been reviewed by Fevold (39). This globulin-like protein occurs widely in egg albumin, tears, saliva, serum, plasma, leucocytes, and organs of the animal body. A simple, efficient method for the purification and crystallization of egg white lysozyme has been reported by Alderton et al. (3, 4). The amino acid composition has been determined by Fromageot et al. (43, 44, 45) and by Lewis (70).

The empirical formula as given by Lewis (70) is Gly_{11}, Ala_{10}, Leu_8, $Ileu_6$, Pro_2, Phe_3, $(CyS-)_{10}$, Met_2, Try_8, Arg_{11}, His_1, Lys_6, Asp_{20}, Glu_4, Ser_{10}, Thr_7, Tyr_3, $Amide_{18}$. A molecular weight of 14,700 ± 250 was reported by Fromageot et al. (45). There is not complete agreement among workers as to the values for Leu, Ileu, and Lys.

Thompson (93), using the dinitrofluorobenzene reagent, demonstrated that lysozyme contained one residue of lysine

at the aminoid end of the protein chain and five other lysine residues. She, also, showed (94), by means of cleavage by carboxypeptidase and chromatography, that leucine was at the carboxoid end of the chain.

Green and Schroeder (50), also by the DNFB method, found a terminal lysine, and Schroeder (85), by the same technique, showed the sequence of four amino acids at the aminoid end of the single polypeptide chain of lysozyme to be lysylvalylphenylalanylglycyl. De Fontaine (34), using a quantitative sequence method (41), confirmed the sequence lysylvalylphenylalanyl.

Acher et al. (2), utilizing partial hydrolysis during eight hours at 37° C. in 11.2 N HCl, chromatography of the resulting peptides, and identification by the DNFB method, were able to shcw the existence of the amino acid sequences, arginylhistidyllysyl, tyrosylglycyl, and glycyltyrosyl.

The isoelectric point of the protein has been reported to be pH 10.8, and the point of greatest solubility, approximately pH 4.5.

Alderton et al. (4) followed destruction of the bacteriolytic properties of lysozyme after divers treatments. Trypsin at pH 7.4 and papain-cysteine at pH 6.0 caused little, if any, destruction of this activity, while pepsin at pH 2.0 caused 56 per cent destruction in the 24 hour period at 30° C. Heating in HCl solution at pH 3.0 at 96° C. for

5 minutes caused no destruction of activity. At the end of 25 minutes, 21 per cent loss in activity was observed, and at the end of 80 minutes, 60 per cent loss. Samples heated likewise to a 40 per cent reduction in activity and treated with proteolytic enzymes showed an increased susceptibility to proteolysis. After removal of the digested part by dialysis, however, the bacteriolytic activity of the undigested remainder was the same as that of the original unheated material. It was concluded that some change in the lysozyme molecule is induced by heating which does not disrupt the structure necessary for lytic activity but which does sensitize the molecule to enzyme action.

Methods for Determining Carboxoid Terminal Residues

Methods for determining amino acid residues bearing free alph-carboxyl groups were reviewed by Fox (40) in 1945. A few new techniques have been introduced since then.

Fromageot et al. (45) have recently made use of lithium aluminum hydride to reduce the free carboxyl groups to primary alcohol groups. After hydrolysis with hydrochloric acid, the amino alcohols thus formed are extracted from the hydrolyzate with ether. They may be separated and identified by paper chromatography. Using this method on insulin,

they were able to identify the amino alcohols derived from glycine and alanine.

Chibnall and Reese (30) reported a similar method in which the protein was first esterified with diazomethane and then reduced with $LiBH_4$. After acid hydrolysis the amino acids and amino alcohols present were treated with periodic acid. The amino alcohols were oxidized to aldehydes with the same side chain as the original amino acids with the production of one mole of formaldehyde for each amino alcohol. At the same time serine and threonine were oxidized to formaldehyde and acetaldehyde, respectively.

When insulin was treated in this manner, the hydrolyzate yielded two equivalents of acetaldehyde and six equivalents of formaldehyde in excess of the amounts that would be given by the threonine and serine present. This indicated that insulin has two free carboxoid groups which are glycine and two which are alanine.

Boissannas (25) has applied anodic oxidation of terminal carboxoid residues of simple peptides to a subtractive sequence study of the two end-most residues. The carbobenzoxy or dinitrophenyl peptide is subjected to anodic oxidation in methanol. An aliquot is evaporated to dryness and completely hydrolyzed with acid. The chromatogram of this material is compared to a chromatogram of an hydrolyzate of the original peptide derivative. The amino acid not

appearing on the chromatogram of the oxidized material is the terminal carboxoid residue.

A second aliquot is subjected to a partial hydrolysis in 20 per cent hydrochloric acid for 15 minutes at 100° C. The solution is repeatedly evaporated under reduced pressure after adding water to remove HCl, and then it is subjected to a second oxidation, hydrolysis, and chromatographic separation. The amino acid disappearing here is the second residue from the carboxoid terminus.

Kenner, Khorana, and Stedman (63) have described a new reagent for the preparation of thiohydantoins involving the carboxoid residues of peptides or amino acid derivatives. To a solution of 2 millimoles of acylpeptide in 15 ml. of anhydrous acetonitrile were added 0.3 ml. (2.2 millimoles) of triethylamine and then 0.64 gm. (2.2 millimoles) of diphenylphosphoroisothiocyanatidate, $(C_6H_5O)_2$ P(O)NCS. The solution was kept at room temperature in a sealed flask for two days and then evaporated in a vacuum. The solution of the residue in ethyl acetate was washed with 2 N HCl and then sodium bicarbonate solution. The ethyl acetate was dried and evaporated, and the residue recrystallized from acetone-petroleum ether.

1-Benzoylglycine-5-isobutyl-2-thiohydantoin thus prepared was shown to hydrolyze to hippuric acid and 5-isobutyl-2-thiohydantoin almost quantitatively in 15 minutes in 0.001 N NaOH.

The recent work of Waley and Watson (98) pointed up the continued interest in the ammonium thiocyanate-acetic anhydride-acetic acid method (58, 59, 84) for derivatizing the carboxoid amino acid residues. Alanine was demonstrated to be one of the carboxoid terminal residues of insulin, by isolating the thiohydantoin from the reaction mixture. Acetyated insulin was heated 30 minutes on a water bath with ammonium thiocyanate in acetic anhydride. The acetic anhydride was removed *in vacuo*, and the last traces were removed by dissolving the residue in water, neutralizing with ammonia, and evaporating in a vacuum desiccator over P_2O_5 and NaOH. The residue was treated with 0.1 N $Ba(OH)_2$ for 15 minutes, and the solution was neutralized with carbon dioxide and extracted with ethyl acetate. The solvent was evaporated, and the extracted thiohydantoins were hydrolyzed with constant boiling hydrobromic acid for three hours at 155° C. The resulting amino acids were identified by chromatography.

Using a similar procedure for thiohydantoin preparation, Tibbs (95) hydrolyzed in 0.25 N $Ba(OH)_2$ for 48 hours at 140° C. and separated chromatographically.

Baptist and Bull (9) have carried out the thiohydantoin formation by reacting 0.1 millimole of synthetic peptide with 1 to 1.5 millimoles of anhydrous ammonium thiocyanate dissolved in 2 ml. of 9:1 acetic anhydride-

acetic acid solution at 40-45° C. for 4 hours with stirring. Two ml. of 20 per cent hydrochloric acid was added dropwise with good agitation. The solution was heated one hour on the steam bath and taken to dryness under vacuum. The thiohydantoin was isolated from a 2.25 M phosphate buffer solution at pH 6.5 by extracting with ethyl acetate. The solvent was removed, and the dry thiohydantoin was hydrolyzed with 1.25 N $Ba(OH)_2$ at 140° C. for 5 hours. The resulting amino acids were determined by filter paper chromatography. By their modification, alanine, phenylalanine, and tyrosine were found as terminal carboxoid residues in insulin (cf. 30, 45).

In the present work a quantitative modification of the Schlack and Kumpf procedure was sought in the main, although a blocking procedure introduced by Dakin and West (33) was attempted. The latter authors described a reaction whereby an amino acid is heated on a water bath in acetic anhydride and pyridine. This causes decarboxylation and transformation of the amino acid into a ketone. In a typical reaction, 3 gm. of phenylalanine, 10 ml. of acetic anhydride, and 10 ml. of pyridine were heated on a steam bath for 5 hours. Carbon dioxide evolved freely in 10 minutes and was almost completely removed in one hour. The reaction is as follows:

$$C_6H_5CH_2CH\ NH_2COOH + (CH_3CO)_2O \xrightarrow{\text{Pyridine}} C_6H_5CH\begin{matrix}COCH_3\\NHCOCH_3\end{matrix} + CO_2$$

EXPERIMENTAL

Materials

Peptides

The preparation and the constants of the peptides used in this study, which had not been recorded previously in the literature, were reported by Fox, Hurst, and Itschner (41).

Lysozyme

The lysozyme used was crystalline egg white lysozyme, lots 003L and 003L1, obtained from Armour and Company, Chicago. Lot 003L gave a homogeneous electrophoretic pattern. The other lot was not checked. Samples for moisture determination and for the proteolytic studies were weighed at the same time. Moisture was determined by drying to constant weight at 105° C. Appropriate corrections were made for the moisture content.

Chemicals and reagents

Chemicals and reagents were of ordinary laboratory reagent or C. P. grade.

Bacterial cultures

The following bacterial cultures were purchased from the American Type Culture Collection and have been used routinely in microbiological assays of amino acids: Lactobacillus arabinosus, ATCC 8014; Leuconostoc mesenteroides, ATCC 8042; Leuconostoc citrovorum, ATCC 8081; Streptococcus faecalis, ATCC 8042; and Lactobacillus brevis, ATCC 8287.

Enzymes

Chymotrypsin was the crystallized enzyme from bovine pancreas and contained less than 50 per cent of $MgSO_4 \cdot 7H_2O$. It was obtained from Armour and Company as lot 90402. No further purification was employed in this study.

Trypsin was the recrystallized enzyme obtained from bovine pancreas and contained less than 50 per cent $MgSO_4 \cdot 7H_2O$. Lot 904C13 from Armour and Company was used as obtained.

Pepsin was obtained from Armour and Company as lot 10428 of crystallized pepsin from porcine mucosa. It was used as obtained.

Papain was obtained from Nutritional Biochemicals Corporation as lot 3781. Before use, 80 mgm. of papain was suspended in 5 ml. of 5 per cent sodium cyanide solution and set in the refrigerator for about 15 hours. The enzyme was resuspended, and a one ml. aliquot was used.

Buffers

The pH 8.5 buffer was made by dissolving 2.10 gm. of $NaHCO_3$ in almost 500 ml. of water, adjusting to pH 8.5 by adding NaOH solution, and diluting to 500 ml. to make an approximately 0.05 M solution.

The pH 5 buffer was prepared by dissolving 3.40 gm. of KH_2PO_4 in nearly 500 ml of water, adjusting to pH 5.0 with dilute hydrochloric acid and diluting to 500 ml. This is approximately 0.05 M. The pH 4 buffer was prepared by dissolving 1.15 ml. of acetic acid in distilled water, adjusting to pH 4 with dilute NaOH solution and diluting to 200 ml. to make an approximately 0.1 M acetate solution.

Methods

Microbiological assays

Microbiological assays were carried out by procedures routinely used. The media used are listed below with the amino acid to be assayed omitted.

Medium A. Same as medium of Kuiken, et al. (65), except that in each liter of medium there were included 40 gm. of sodium acetate, 470 mgm. of L-glutamic acid, 300 mgm. of L-lysine hydrochloride, 800 mgm. of DL-tryptophan, 400 mgm. of L-tyrosine, 2000 micrograms of thiamine chloride

hydrochloride, 400 micrograms of pyridoxine hydrochloride, 400 micrograms of calcium pantothenate, 10 micrograms of biotin, and 400 micrograms of p-aminobenzoic acid, instead of the corresponding amounts or forms listed. In addition were included 400 mgm. of DL-norleucine, 400 mgm. of glycine, 400 micrograms of folic acid, and no tomato eluate preparation.

Medium B. Same as Medium A with the inclusion of 40 ml. of Salt A solution (83).

Medium C. Same as Medium B with only 50 mgm. of aspartic acid and 25 mgm. of glutamine per liter of medium (71).

Medium D. Same as Medium A with substitution of 10 gm. of arabinose for 10 gm. of glucose (38).

Medium E. Medium VI of Steele, et al. (91) with 100 mgm. of DL-serine and 40 mgm. of glycine per liter and no pyridoxamine or pyridoxal.

Medium F. Medium of Kuiken, Lyman, and Hale (64).

Medium G. Medium II of Sauberlich and Baumann (83) with half as much of each purine.

Standard curves covered the range of 0-50 micrograms of L-amino acid. The final pH of media prior to inoculation was 6.8-7.0 in each case except when arabinose (pectin sugar) was employed. In these cases pH was 6.5; the lower pH minimized darkening due to autoclaving. In-

cubation was for 72 hours at 37° C., except for glycine assays in which incubation was stopped at 48 hours. The standard alkali was 0.05 N NaOH solution, the endpoint being observed with bromthymol blue.

Table 1 summarizes the microorganism and medium used for each amino acid determined.

Samples for assay were usually set up in triplicate at two levels. The levels were chosen so that the probable assay values would lie between 10 and 30 micrograms. Aliquots used were usually whole numbers to help eliminate errors in reading pipettes, and the second level was twice the volume of the first. In each case the sample aliquot was diluted to 2.5 ml. by adding water, and 2.5 ml. of the appropriate medium was added to give a total volume of 5.0 ml. in each tube.

Each point obtained by titrating the standard, which was also set up in triplicate for each level, was plotted, and the best curve passing through these points was drawn. The micrograms of amino acid corresponding to the titration values for the samples were read from this curve. Titration values for the replications which varied more than 20-30% from the others were usually discarded. The remaining values were averaged. If there was not close agreement within replications, all titration values were averaged and these values read from the graph. If the two assay levels

Table 1

Microorganisms and Media for Microbiological Assays

Amino Acid	Microorganism	Medium
Arginine	S. faecalis	A
Aspartic Acid	L. mesenteroides	B
Glutamic Acid	L. arabinosus	C
Glycine	L. brevis	D
Histidine	L. mesenteroides	A
Isoleucine	L. arabinosus	A
Leucine	L. arabinosus	A
Lysine	S. faecalis	A
Methionine	S. faecalis	F
Phenylalanine	L. brevis	D
Proline	L. brevis	D
Serine	L. mesenteroides	E
Threonine	S. faecalis	G
Tyrosine	L. brevis	D
Valine	S. faecalis	A

gave values varying by more than 10 per cent from the larger, the determination was repeated.

In some assays growth response curves for samples were not similar to the standard curve. In these instances aberrant values were not used.

Chromatographic identifications

Qualitative identifications of the amino acids were performed by one dimensional ascending paper chromatography in butanol-acetic acid-water, 4:1:5, solvent (76). Whatman No. 1 or No. 4 filter paper was used. After drying, the paper was sprayed with a 0.2 per cent ninhydrin solution in n-butanol saturated with water or dipped in a 0.1 per cent ninhydrin solution in acetone. The paper was air dried and then placed in the oven at 105° C. for five minutes. Identifications were made by running controls simultaneously.

Carboxoid residue blocking experiments

Initial trials. Two mgm. portions of leucylvaline, valylleucine, and L-prolyl-L-leucine were heated in weighing bottles with 1 mgm. portion of ammonium thiocyanate and 2 ml. portions of acetic anhydride for one-half hour on the water bath.

The acetic anhydride was evaporated off over H_2SO_4 and

and NaOH in a vacuum desiccator, and the residues were treated with 2 ml. of concentrated NH_4OH for 4 hours at room temperature. The NH_4OH was then evaporated off in the vacuum desiccator.

To each bottle 2 ml. of 2 N HCl were added, and the bottles were then autoclaved at 15 pounds pressure for four hours. The HCl was evaporated on the water bath, and the contents of each bottle were made to 50 ml. Results of the microbiological assay as per cent recovery follow:

Leucylvaline			
Leucine	75%	Valine	4%
Valylleucine			
Leucine	31%	Valine	80%

The amino acids of L-prolyl-L-leucine were not determined microbiologically, but a chromatogram using butanol-acetic acid as solvent indicated considerable blocking of the leucine.

Similar tests were made with leucylvaline and valylleucine using 1, 2, 3, and 4 hours' treatment with concentrated NH_4OH followed by a 16 hour hydrolysis with 6 N HCl at 120° C. The results of these tests appear in Table 2.

Two mgm. of valylglycylphenylalanine in a weighing bottle were treated with 2 ml. of acetic anhydride and 4 mgm. of NH_4SCN on the water bath for one hour. The content of the bottle was evaporated to dryness in a vacuum desiccator containing a beaker of flake NaOH and one of H_2SO_4. Two ml. of

concentrated NH_4OH were then added. The bottle was rotated to dissolve the residue and allowed to stand one hour at room temperature. The ammonia was then cautiously evaporated in the vacuum desiccator.

Table 2

Microbiologically Recoverable Amino Acids from Carboxoid Blocked and Ammonia Treated Dipeptides

Dipeptide	Hours of Ammonia Treatment	Per Cent Recovery	
		Leucine	Valine
Leucylvaline	1	73	33
	2	76	8
	3	75	28
	4	26	28
Valylleucine	1	32	104
	2	22	65
	3	27	37
	4	10	37

The residue was then hydrolyzed with 2 ml. of 2 N HCl for 12 hours. After evaporation on the water bath, neutralization, and dilution, the microbiological assays were run for valine, glycine, and phenylalanine. Results of the microbiological assays are as follows:

Valine 98% Glycine 117% Phenylalanine 6%

Two mgm. of leucylglycylvaline were treated exactly as for valylglycylphenylalanine above with the following results:

Leucine 83% Glycine 97% Valine 4%

Another series of tests were set up on L-prolyl-L-leucine with several variations.

PL-1. Five mgm. of L-prolyl-L-leucine in a weighing bottle were treated with 3 ml. of acetic anhydride and 1 ml. of dioxane-HCl. After 10 minutes at room temperature, 10 mgm. of NH_4SCN were added. A white precipitate, probably NH_4Cl, formed at once. After 4 hours at room temperature, the weighing bottle was set in a vacuum desiccator and evaporated to dryness in about 24 hours. Then 1 ml. of concentrated NH_4OH was added, and, after 20 minutes at room temperature, the weighing bottle was set in a vacuum desiccator, and the contents were evaporated to dryness. Two ml. H_2O were added to the residue. In these runs the residues were not acid hydrolyzed.

PL-2. Five mgm. of L-prolyl-L-leucine were heated on a water bath for 10 minutes with 2 ml. of acetic anhydride. Then 10 mgm. of NH_4SCN were added, and the heating was continued for 50 minutes. The content of the bottle was evaporated to dryness. The residue was treated with 1 ml. of concentrated NH_4OH for one hour, and the experiment was completed as for PL-1.

PL-3. Five mgm. of L-prolyl-L-leucine were treated with 2 ml. of acetic anhydride and 0.1 ml. of concentrated HCl for 10 minutes at room temperature. Then 10 mgm. of NH_4SCN were added, and the bottle was allowed to stand one hour at room temperature. The experiment was completed as for PL-1.

PL-4. The experiment was the same as PL-3 except that 0.1 ml. of 48% HBr was used instead of HCl.

PL-5. The experiment was the same as PL-3 except that 0.5 ml. of dioxane-HCl was used instead of 0.1 ml. of concentrated HCl.

PL-6. Five mgm. of L-prolyl-L-leucine were treated with 0.05 ml. of 48% HBr and 2 ml. of acetic anhydride at 100° C. for 5 minutes. Then 10 mgm. of NH_4SCN were added, and the bottle was heated 10 minutes longer. The experiment was completed as above.

PL-7. Five mgm. of L-prolyl-L-leucine were treated with 2 ml. of dioxane-HCl and 10 mgm. of NH_4SCN for 2 hours at room temperature. The experiment was completed as above. Results of the microbiological assays on these samples which were not acid hydrolyzed appear in Table 3.

Five mgm. portions of leucylvaline, valylleucine, valylglycylphenylalanine and leucylglycylvaline were each treated with 2 ml. of acetic anhydride and 0.5 ml. of dioxane-HCl and 10 mgm. of NH_4SCN. After standing 1.5 hours

at room temperature, the bottles were placed in a vacuum desiccator and evaporated. Two ml. of concentrated NH_4OH were added and allowed to react one hour. The ammonia was evaporated *in vacuo*, 2 ml. of 2 N HCl were added, and the bottles were autoclaved 12 hours at 120° C. The contents of the bottles were evaporated to dryness on the water bath.

Table 3

Per Cent Recovery of Amino Acids in Treated Prolylleucine

Experiment	L-Proline	L-Leucine
PL-1	9	44
PL-2	0	27
PL-3	16	77
PL-4	21	54
PL-5	12	35
PL-6	8	41
PL-7	40	93
Peptide	88	--

After adjustment of the pH, the volumes were made to 100 ml. Chromatograms showed little blocking.

The microbiological recoveries were as follows:

Valylglycylphenylalanine
Valine 83% Phenylalanine 35%

Valylleucine
Valine 40% Leucine 26%

Five ml. portions of the above four peptides equivalent to 5 mgm. were each treated with 5 ml. of pyridine containing 0.005 ml. of benzoyl chloride. The reaction was kept at pH 7-8 by adding 0.2 N NaOH and allowed to proceed for 3 hours at 37° C. The solutions were evaporated to dryness *in vacuo*.

To each were added 2 ml. of acetic anhydride, 0.1 ml. of dioxane-HCl and 10 mgm. of NH_4SCN. The bottles were heated 30 minutes at 100° C. After evaporating *in vacuo* the contents were treated for 1 hour with 2 ml. of NH_4OH at room temperature. Following evaporation the contents were hydrolyzed 12 hours with 2 ml. of 2 N HCl. After evaporation on the water bath the materials were diluted to 100 mls. Chromatograms showed partial blocking of carboxyl residues.

The microbiological assays were as follows:

Valylglycylphenylalanine			
Valine	79%	Phenylalanine	10%
Valylleucine			
Valine	64%	Leucine	71%
Leucylglycylvaline			
Leucine	50%	Valine	20%

Two mgm. of leucylglycylvaline and valylglycylphenylalanine and 2.5 mgm. of L-prolyl-L-leucine each in 2 ml. of H_2O were each treated with 2 ml. of pyridine containing 0.002 ml. of benzoylchloride as above. After evaporation, 0.5 ml. of acetic anhydride, 1.0 ml. of dioxane-HCl and about 5 mgm. of NH_4SCN were added. After standing 12 hours at room temper-

ature, the solutions were evaporated *in vacuo*, and then the residues were treated with 2 ml. of NH_4OH for 2 hours. After evaporation hydrolysis was carried out with 2 ml. of N HCl for 12 hours. The solutions were then evaporated on the water bath. After adjustment of the pH the volumes were made to 50 ml.

The microbiological values follow:

L-Prolyl-L-leucine
Proline 60% Leucine 17%
Valylglycylphenylalanine
Valine 83% Phenylalanine 14%
Leucylglycylvaline
Valine 23%

Four 2 ml. (2 mgm.) portions of L-prolyl-L-leucine were pipetted into weighing bottles, and 2 ml. of pyridine containing 0.003 ml. of benzoyl chloride were added to each. The pH was maintained at 7-8 by adding 0.2 N NaOH. The reaction was allowed to go for 3 hours at 37° C. After evaporation *in vacuo*, about 4 mgm. of NH_4SCN and 2 ml. of acetic anhydride containing 10% acetic acid were added.

Bottle 1 was heated 30 minutes on the boiling water bath. After evaporation *in vacuo*, 2 ml. of concentrated NH_4OH were added and allowed to stand at room temperature for 2 hours. The ammonia was evaporated *in vacuo*, and 2 ml. of 6 N HCl were added. The material was hydrolyzed in the usual manner.

Bottle 2 was treated as Bottle 1 except for the use of 2 ml. of 0.1 N NaOH for 30 minutes at room temperature

instead of NH_4OH for 2 hours. Two ml. of 12 N NCl were then added and autoclaved to hydrolyze.

Bottle 3 was heated 60 minutes on the boiling water bath and treated as Bottle 1.

Bottle 4 was heated 60 minutes on the boiling water bath and then treated as Bottle 2.

Microbiological assays were made on these samples. Results appear in Table 4.

Table 4

Microbiological Recoveries of Amino Acids in Treated Prolylleucine

Bottle	Treatment	Per Cent Recovery	
		Proline	Leucine
1	30 minutes on water bath 2 hours with NH_4OH	68	16
2	30 minutes on water bath 30 minutes with 0.1 N NaOH	77	12
3	60 minutes on water bath 2 hours with NH_4OH	61	4
4	60 minutes on water bath 30 minutes with 0.1 N NaOH	67	22

Two mgm. of leucylglycylvaline were heated on the water bath for 10 minutes with 2 ml. of acetic anhydride containing 10% acetic acid. Then about 3 mgm. of NH_4SCN were added, and the heating was continued for 50 minutes.

After evaporation to dryness in a vacuum desiccator, 2 ml. of NH_4OH and 0.1 ml of 0.1 N NaOH were added and allowed to react for about 4 hours. Evaporation of ammonia was done in a vacuum desiccator over H_2SO_4. After drying, 2 ml. of 6 N HCl were added and hydrolysis was carried out at 120° C. for 16 hours. Microbiological assays of the resulting solution and solutions of other peptides treated similarly gave the following results:

Leucylglycylvaline					
	Leucine 93%	Glycine 95%		Valine 2%	
Leucylvaline					
	Leucine 68%	Valine 24%			
Valylleucine					
	Valine 45%	Leucine 12%			
L-Prolyl-L-leucine					
	Proline 60%	Leucine 16%			
Valylglycylphenylalanine					
	Valine 67%	Glycine 70%		Phenylalanine 3%	

Two mgm. of leucylvaline were heated on a water bath in a weighing bottle for 10 minutes with 2 ml. of acetic anhydride. Then 0.02 ml. (6 mgm.) of NH_4SCN in CH_3OH were added, and the heating was continued for 50 minutes longer. After evaporation of the acetic anhydride, the residue was treated with 2 ml. of concentrated NH_4OH for 4 hours. The ammonia in turn was evaporated, and the residue was hydrolyzed with 2 ml. of 6 N HCl for 12 hours at 120° C. Results for this peptide and others treated likewise after chromatographing are as follows:

Leucylvaline - partial blocking of valine
Valylleucine - partial blocking of valine
Valylglycylphenylalanine - complete blocking of phenylalanine

Two mgm. of valylleucine and leucylvaline were treated as in the experiment directly above. Chromatograms were spotted after ten minutes acetylation. No ninhydrin positive spots were obtained after developing. Apparently the acetylation was complete.

A chromatogram of the material after 50 minutes' heating with NH_4SCN gave no ninhydrin positive spot.

The spots made after 1/2, 1, 2, and 3 hours treatment with ammonia were negative except for the 1/2 hour treatment. In this case faint spots were obtained which were not the original peptides or amino acids. After evaporation of the NH_4OH the residues were treated with 2 ml. of 0.1 N NaOH. A chromatogram spotted after 15, 30, and 45 minutes gave spots which were barely above the base line and appeared progressively more intense.

After hydrolysis with 6 N HCl at 120° C. for 12 hours, positive tests were obtained for leucine and valine.

Even though autoclaving the thiohydantoic acid may reform the thiohydantoin, it must later be considerably if not completely hydrolyzed under these conditions.

A test similar to the above was carried out with the omission of the NH_4OH step and addition of 0.1 N NaOH to a pH of 7.5-8. Hydrolysis was carried out with 2 N HCl for 12 hours at 120° C. Results were identical to those above except that some free peptide was obtained for each, besides leucine and valine.

Leucine and phenylalanine were treated as above with similar results.

It appeared that thiohydantoins without substituents on the N_1 or N_3 group were labile to acid hydrolysis.

The lability of the thiohydantoin of leucine is exemplified by the following experiment. One mgm. portions of 5-isobutyl-2-thiohydantoin, prepared from DL-leucine and NH_4SCN by the method of Johnson (58) were placed in four 14 x 100 mm. test tubes and in four 50 ml. beakers.

Four ml. of 3 N HCl were placed in Test Tube 1 and Beaker 1. Four ml. of 4 N HCl were placed in Test Tube 2 and Beaker 2. Likewise, four ml. of 6 N and 8 N HCl were pipetted into Test Tubes 3 and 4 and Beakers 3 and 4. These test tubes were sealed by drawing out in a flame, the beakers were covered by petri dishes, and all were autoclaved for 16 hours at 120° C.

The liquid volumes in the beakers increased by the condensation of steam and amounted to 10 ml. for Beakers 1, 2, and 3, and 17 ml. for Beaker 4. Thus, before the end of the hydrolysis the concentration of the HCl had been reduced to less than 2.5 N.

The tubes were opened and their contents transferred to 50 ml. beakers. All solutions were then evaporated on a water bath, diluted to 50 ml. and assayed for leucine equivalent by the microbiological procedure.

The results appear in Table 5.

Table 5

Microbiologically Available Leucine From 5-Isobutyl-2-Thiohydantoin After Acid Treatment

	Initial HCl Normality	% Leucine Equivalent
Test Tube 1	3	30
" " 2	4	39
" " 3	6	58
" " 4	8	57
Beaker 1	3	41
" 2	4	42
" 3	6	35
" 4	8	30
Non-acid Treated Thiohydantoin		30-60

It can be seen that the thiohydantoin of leucine furnishes microbiologically available leucine or its equivalent. This would account for the apparent recoveries of blocked carboxoid residues, which in many cases amounts to 30-35%. It is probable that only the 6 N and 8 N HCl in sealed test tubes causes hydrolysis of the thiohydantoin into leucine. This could be determined by chromatographing such hydrolyzates.

Another sample of the thiohydantoin prepared from DL-leucine was treated for 30 minutes with 1:1 NH_4OH:H_2O. The solution was dried in a vacuum desiccator and hydrolyzed

with 3 N HCl for 16 hours at 120° C. After evaporation and dilution, the microbiological assay showed a recovery of only 10% of the theoretical amount of L-leucine in the thiohydantoin.

Effect of acids on the ammonium thiocyanate blocking reaction. The following experiments were run to determine the effect of added acid on the ammonium thiocyanate blocking reaction:

Treatment 1. In a small weighing bottle 2.6 mgm. of benzoyl-DL-leucylglycyl-DL-phenylalanine, 2 ml. of formic acid, and 1 ml. of acetic anhydride were heated 30 minutes on a boiling water bath. Solvents were evaporated in a vacuum desiccator, and the residue was hydrolyzed with 5 ml. of 6 N HCl. After evaporation and dilution, the microbiological assays were run with the following results:

Leucine 72% Phenylalanine 82%

Treatment 2. The treatment above was applied to 2.2 mgm. of DL-leucylglycyl-DL-phenylalanine with the following results:

Leucine 95% Phenylalanine 76%

Likewise, using 20 per cent acetic acid in the acetic anhydride in the usual procedure, little blocking of the carboxoid residue in valylglycylphenylalanine or in leucylglycylvaline was obtained, as determined by chromatographing hydrolyzates of the treated peptides. From using 20 per cent

dichloroacetic acid in the acetic anhydride, partial blocking of the carboxoid residue of valylglycylphenylalanine was apparent but not so with leucylglycylvaline. Five per cent dichloroacetic acid and 5 per cent hydrochloric acid in the acetic anhydride, likewise gave no blocking.

Effect of prior blocking of the aminoid terminus by benzoyl group on the ammonium thiocyanate blocking reaction. Two mgm. of valylglycylphenylalanine in 2 ml. of H_2O were treated with 2 ml. of pyridine containing 0.04 ml. of benzoylchloride and a little bromthymol blue. The reaction was allowed to proceed for 6 hours at 37° C. with frequent adjustment of pH with 0.2 N NaOH.

After evaporation in vacuo, 2 ml. of acetic anhydride containing 10% acetic acid and 30 mgm. of NH_4SCN in 0.1 ml. of CH_3OH were added. The bottle was heated 30 minutes on the boiling water bath and then evaporated in vacuo. The material was hydrolyzed with 4 ml. of 3 N HCl for 15 hours at 120° C. After evaporation, neutralization, and dilution to volume the microbiological tests were set up. The results follow:

Valylglycylphenylalanine
Valine 72% Glycine 90% Phenylalanine 6%

Two ml. containing 2 mgm. of leucylglycylvaline were placed in a small weighing bottle. Two ml. of pyridine containing 0.002 ml. benzoyl chloride and a little bromthymol blue were added. The pH was maintained at 7.5-8 by adding

0.2 N NaOH. The reaction was allowed to proceed for 3 hours at 37° C. with periodic adjustment of pH. The liquid was then evaporated in a vacuum desiccator over H_2SO_4 and NaOH.

Two ml. of acetic anhydride containing 10% acetic acid and about 4 mgm. of NH_4SCN were added. The weighing bottle was closed and heated on the boiling water bath for 30 minutes. The contents of the bottle were then evaporated to dryness in the vacuum desiccator.

Two ml. of 0.1 N NaOH were added to the bottle and allowed to react 3 hours at room temperature. Then 2 ml. of 12 N HCl were added, and the bottle was set in the autoclave at 120° C. for 16 hours. Evaporation of the HCl was done on the water bath. The pH was adjusted and volume made to 50 ml.

Valylglycylphenylalanine, leucylvaline, valylleucine, and L-prolyl-L-leucine were treated in like manner.

Results of the microbiological assays as per cent recovery are as follows:

Leucylglycylvaline		
Leucine 83%	Glycine 98%	Valine 33%
Valylglycylphenylalanine		
Valine 64%	Glycine 88%	Phenylalanine 11%
L-Prolyl-L-leucine		
Proline 67%	Leucine 2%	
Leucylvaline		
Leucine 77%	Valine 29%	
Valylleucine		
Valine 28%	Leucine 3%	

A comparison of a benzoylated and non-benzoylated tripeptide was made as follows:

Experiment 1. A 1 ml. aliquot of an acetic acid solution of benzoyl DL-leucyl-DL-phenylalanine (equivalent to 2.6 mgm.) in a small weighing bottle was evaporated in a vacuum desiccator over NaOH and H_2SO_4. Four mgm. of NH_4SCN and 2 ml. of acetic anhydride containing 10 per cent acetic acid were added and heated 30 minutes on a boiling water bath. The solvent was evaporated in a vacuum desiccator, and the residue was hydrolyzed with 5 ml. of 6 N HCl for 16 hours. The experiment was completed and the microbiological assay was run. The results were:

Benzoylleucylphenylalanine
Leucine 89% Phenylalanine 26%

Experiment 2. This experiment was run as Experiment 1 except that after the NH_4SCN-acetic anhydride treatment and evaporation, 1 ml. of 0.1 N NaOH (cf. 80) was added and allowed to stand 10 minutes before the 5 ml. of 6 N HCl was added. These results were:

Benzoylleucylphenylalanine

Leucine 76% Phenylalanine 30%

Experiment 3. This was performed as Experiment 1 except 2.2 mgm. of DL-leucylglycyl-DL-phenylalanine were used. The assays showed recoveries of:

Leucylglycylphenylalanine
Leucine 96% Phenylalanine 29%

Experiment 4. This was performed as Experiment 2 except 2.2 mgm. of DL-leucylglycyl-DL-phenylalanine was used. The recoveries were:

Leucylglycylphenylalanine
Leucine 97% Phenylalanine 31%

Effect of prior blocking of the aminoid terminus by phenylisothiocyanate on the ammonium thiocyanate blocking reaction. Two mgm. of leucylvaline in 2 ml. of H_2O were treated with 2 ml. of pyridine containing a little bromthymol blue and about 30 mgm. of phenylisothiocyanate. A dark green color was maintained during 5 hours' incubation at 37° C. by adding, at intervals, small amounts of 0.2 N NaOH. Following evaporation in vacuo, the material in a closed weighing bottle was heated on a boiling water bath for 30 minutes with 2 ml. of acetic anhydride containing 10% acetic acid. This liquid was then evaporated in vacuo, and the residue was hydrolyzed with 3 N HCl for 15 hours at 120° C. The acid was removed by evaporating on the water bath, the pH was adjusted, and the volume made to 50 ml. A microbiological assay gave the following results:

Leucylvaline
Leucine 4% Valine 53%

Two mgm. of valylglycylphenylalanine were first treated with phenylisothiocyanate in the usual manner. After evaporation of the aqueous pyridine, the residue was treated with 2 ml. of acetic anhydride and 4 mgm. of NH_4SCN on the

water bath for one hour. The content of the bottle was evaporated to dryness in a vacuum desiccator containing a beaker of flake NaOH and one of H_2SO_4. Two ml. of concentrated NH_4OH were then added. The bottle was rotated to dissolve the residue and was allowed to stand one hour at room temperature. The ammonia was then cautiously evaporated in the vacuum desiccator.

The residue was then hydrolyzed with 2 ml. of 2 N HCl for 12 hours. After evaporation on the water bath, neutralization, and dilution, the microbiological assay was run for valine, glycine, and phenylalanine. The results were:

Valylglycylphenylalanine
Valine 0% Glycine 77% Phenylalanine 15%

Comparisons of no-ammonia, ammonia, and ammonia plus hydrogen peroxide treatments following the ammonium thiocyanate reaction. These comparisons were performed as follows:

Treatment 1. Two mgm. each of glycyl-DL-phenylalanine, benzoylglycyl-DL-phenylalanine, and DL-leucylglycyl-DL-phenylalanine in separate weighing bottles were heated on a water bath 30 minutes with 30 mgm. of NH_4SCN and 5 ml. of acetic anhydride containing 10 per cent acetic acid. After the bottles had cooled somewhat, 1 ml. of H_2O was added to each, the contents were mixed, and the bottles set in a vacuum desiccator over NaOH and H_2SO_4 to evaporate. After the liquid had evaporated, 1 ml. of H_2O and 0.1 ml. of 3 per cent H_2O_2 were added, and the bottles allowed to stand

about 3 hours before setting into the desiccator to evaporate again. Five ml. of 3 N HCl were added to each of the dry bottles, and the bottles were autoclaved for 12 hours at 120° C. The HCl was evaporated on a water bath. The contents of the bottles were diluted, neutralized and diluted to 250, 200, and 200 ml. respectively. Microbiological assays were run.

Treatment 2. The same amounts of the three compounds were treated exactly as for Treatment 1 through the evaporation of the acetic anhydride-acetic acid. Then 1 ml. of 1:1 NH_4OH-H_2O was added to each and allowed to stand 30 minutes before setting in the vacuum desiccator to dry. Acid hydrolysis, dilution, and assays were carried out as before.

Treatment 3. The same amounts of the three compounds were treated exactly as for Treatment 1 through the evaporation of acetic anhydride-acetic acid. Then 1 ml. of 1:1 NH_4OH-H_2O and 0.1 ml. of 3 per cent H_2O_2 were added to each. These were allowed to react 30 minutes and then set in a vacuum desiccator to evaporate. The hydrolysis, dilution, and assays were carried out as before.

The results of the assays are shown in Table 6. Values are given as percentages of the theoretical amount present that is recovered.

Treatments 1 and 3 were also carried out on samples of glycyl-L-proline. No blocking of the proline residue took

place. This was expected since earlier workers (58, 59) had shown that a free hydrogen must remain on the nitrogen atom of the amino acid in order to allow cyclization to the thiohydantoin. As a carboxoid residue of a peptide, proline does not possess such an hydrogen atom on its nitrogen atom.

Table 6

Percentages of Amino Acids Recovered Following Various Treatments

Peptide	Amino Acid	Per Cent Recovery		
		Treatment		
		1	2	3
Glycylphenylalanine	Gly	95	95	97
	Phe	34	18	12
Benzoylglycylphenylalanine	Gly	91	90	--
	Phe	35	12	--
Leucylglycylphenylalanine	Leu	89	105	100
	Gly	94	99	95
	Phe	21	12	2

Carboxoid blocking reaction involving the use of phenylisothiocyanate. Two mgm. of valylglycylphenylalanine in 2 ml. of H_2O were treated with 2 ml. of pyridine containing 0.04 ml. of benzoyl chloride and a little bromthymol blue. The reaction was allowed to proceed for 6 hours at 37° C. with frequent adjustment of pH with 0.2 N NaOH.

After evaporation in vacuo, 2 ml. of acetic anhydride

containing 10% acetic acid and 0.05 ml. of phenylisothiocyanate were added. The bottle was heated 30 minutes on the boiling water bath, and then the liquid was evaporated *in vacuo*. The material was hydrolyzed with 4 ml. of 3 N HCl for 15 hours at 120° C. After evaporation, neutralization, and dilution to volume, the microbiological tests were set up. The results were:

Valylglycylphenylalanine
Valine 81% Glycine 83% Phenylalanine 46%

Two mgm. of valylglycylphenylalanine in 2 ml. of H_2O were treated with 2 ml. of pyridine containing 0.04 ml. of phenylisothiocyanate and a little bromthymol blue. The reaction was allowed to proceed for 6 hours at 37° C. with frequent adjustment of pH with 0.2 N NaOH.

After evaporation *in vacuo*, 2 ml. of acetic anhydride containing 10% acetic acid and 0.05 ml. of phenylisothiocyanate were added. The bottle was heated 30 minutes on the boiling water bath, and then the contents were evaporated *in vacuo*. The material was hydrolyzed with 4 ml. of 3 N HCl for 15 hours at 120° C. After evaporation, neutralization, and dilution to volume, the microbiological tests were set up. The results were:

Valylglycylphenylalanine
Valine 10% Glycine 63% Phenylalanine 45%

Two mgm. of dry valylglycylphenylalanine were heated with 2 ml. of acetic anhydride containing 0.05 ml. of

phenylisothiocyanate for 30 minutes on a boiling water bath. The liquid was evaporated in vacuo, and the residue was hydrolyzed with 3 N HCl for 16 hours at 120° C. After evaporation, neutralization, and dilution the microbiological tests were run. The results were:

Valylglycylphenylalanine
Valine 97% Glycine 52% Phenylalanine 3%

The Dakin-West reaction. A 2 mgm. portion of leucylvaline, 1 ml. of acetic anhydride, and 0.2 ml. of pyridine were heated for one hour on a boiling water bath. The acetic anhydride was evaporated off in a vacuum desiccator, and the residue was hydrolyzed with 6 N HCl for 16 hours. After evaporation the material was chromatographed. Almost complete blocking of valine was indicated. Leucine was still present.

Another 2 mgm. portion of leucylvaline was heated on a boiling water bath with 1 ml. of acetic anhydride for 15 minutes. Two-tenths ml. of pyridine were added, and the heating was continued 45 minutes. The material was evaporated, hydrolyzed, and chromatographed and again showed nearly complete disappearance of valine only.

A third portion heated for 1.5 hours gave only a very faint spot for leucine, thereby indicating that long heating leads to destruction of other residues besides the terminal carboxoid residue.

Initial study of the extent of enzymic hydrolysis of lysozyme

An attempt was made to follow the extent of enzymic hydrolysis by the Van Slyke amino nitrogen method. However, at least a 50 mgm. sample of lysozyme is required to furnish a N_2 volume of even 0.5 ml. At these low readings the results were too erratic, and, since it was desirable to conserve material, another method was sought.

The method described by Kunitz (66) for following hydrolysis of yeast nucleic acid by nuclease was tried. Readings were erratic, sometimes increasing, sometimes decreasing with time. Largest increases were obtained because of formation of precipitates. Greatest differences were noted at wavelength of 280 millimicrons.

Also, the method of J. R. Spies (90), in which optical density of the copper complex formed with amino acids is an index of the amount of hydrolysis, was tried. Good curves were obtained with pure alanine, but the toluene added as a preservative in the enzyme runs contributed most to the optical density at 230 millimicrons.

Either or both of these methods might be workable with proper modifications. Nevertheless, next was tried the precipitating of the coagulable protein in an aliquot of enzymic hydrolyzate by 5% trichlorocetic acid and reading of the optical density of the supernatant at 280 millimicrons (67).

The following solutions were prepared:

Chymotrypsin pH 8.5

60 mgm. of lysozyme
10 ml. of 0.05 M $NaHCO_3$ buffer at pH 8.5
1.2 mgm. of chymotrypsin

Papain pH 8.5

60 mgm. of lysozyme
10 ml. of 0.05 M $NaHCO_3$ buffer at pH 8.5
0.05 ml. of a solution made by mixing 200 mgm. papain in 4 ml. of 5% NaCN and centrifuging

Papain pH 5.0

60 mgm. of lysozyme
10 ml. of 0.05 M KH_2PO_4 buffer at pH
0.05 ml. of papain solution

Trypsin pH 8.5

60 mgm. of lysozyme
10 ml. of $NaHCO_3$ buffer at pH 8.5
1.2 mgm. of trypsin

These solutions were made up in 50 ml. Erlenmeyer flasks. A few drops of toluene were added to each as a preservative, and the flasks were set in the incubator at 37° C.

At intervals 0.5 ml. of the hydrolyzates were pipetted into test tubes and 9.5 ml. of 5.25% trichloracetic acid were added. The tubes were shaken and then centrifuged. The optical densities of the supernatant solutions were read in a Beckman DU Spectrophotometer at 280 millimicrons. The readings are given in Table 7.

Table 7

Optical Densities of Proteolytic Supernatants

Hours of Digestion	Enzyme Employed			
	Chymotrypsin pH 8.5	Papain pH 8.5	Papain pH 5.0	Trypsin pH 8.5
0	.174	.182	.180	.170
5	.252	.137	.136	.227
13	.412	.140	.142	.258
29	.602	.134	.135	.249
53	.722	.146	.127	.264
97	.868	.140	.103	.319
149	.809	.199	.104	.360
173	.838	.212	.109	.396
197	.861	.219	.136	.432
221	.867	.169	.074	.359
245	.875	.225	.135	.433

If these data are plotted, it is seen, for chymotrypsin and trypsin at least, that there is an initially rapid hydrolysis which gradually tapers off up to the 120 hour time. From this point on the change is very gradual.

Proteolysis of lysozyme

From the preliminary study it was decided to remove samples of the enzymic hydrolyzates of lysozyme at 24, 48,

120, and 240 hours for the determination of terminal residues.

Preparation of solutions. The following solutions were prepared:

Chymotrypsin pH 8.5

850 mgm. of lysozyme (Armour Laboratories Lot 003L)
16 mgm. of crystallized chymotrypsin
87.0 ml. of pH 8.5 buffer (0.05 M $NaHCO_3$ adjusted to pH 8.5 with 0.2 N NaOH)

Papain pH 5

850 mgm. of lysozyme
16 mgm. of papain (80 mgm. of papain suspended in 5 ml. of 5% NaCN, shaken, allowed to stand in the refrigerator overnight, resuspended, and 1 ml. used)
84 ml. of 0.05 M KH_2PO_4 buffer adjusted to pH 5 with HCl

Papain pH 7.5

850 mgm. of lysozyme
16 mgm. of papain (prepared as above)
84 ml. of 0.05 M $NaHCO_3$ of pH 7.5

Papain pH 8.5

850 mgm. of lysozyme
16 mgm. of papain (prepared as above)
84 ml. of 0.05 M $NaHCO_3$ at pH 8.5

Trypsin pH 8.5

850 mgm. of lysozyme
32 mgm. of trypsin
85 ml. of 0.05 M $NaHCO_3$ at pH 8.5

Pepsin pH 4

1.0000 gm. of lysozyme (Armour Laboratories Lot 003L1)
20 mgm. of pepsin
100 ml. of 0.1 M acetate at pH 4.

Five-tenths of a ml. of toluene were added to each sample which was contained in a 125 ml. glass stoppered Erlenmeyer flask, and the flasks were set in the incubator at 37° C.

Table 8 shows pH changes during the proteolyses.

Table 8

pH Changes of Reaction Mixture
During Proteolysis of Lysozyme

Treatment	Hours				
	0	24	48	120	240
Chymotrypsin pH 8.5	8.3	8.0	8.2	8.4	8.4
Papain pH 5	5.2	6.5	6.1	6.6	6.6
Papain pH 7.5	7.6	8.9	8.7	8.7	9.3
Papain pH 8.5	8.3	9.1	9.0	9.1	9.3
Trypsin pH 8.5	8.3	8.7	8.4	9.4	9.5
Pepsin pH 4	4.0	4.0	3.7	4.5	4.6

At the designated intervals 5 ml. aliquots were withdrawn and treated by the aminoid residue and the carboxoid residue blocking procedures.

Method for blocking the aminoid terminal amino acid residues. To a 5.00 ml. (approximately 50 mgm. of lysozyme) aliquot of the lysozyme proteolyzate placed in a 50 ml. beaker were added 5 ml. of a solution of 80 ml. of pyridine and 3.2 ml. of phenylisothiocyanate. A small amount of powdered bromthymol blue and 0.2 N NaOH were added to give an emerald green color. The pH was maintained by periodically adding alkali over the 8 hour period in which the reaction was carried out at 37° C. At the end of the 8 hour period, the beaker was set in a vacuum desiccator over NaOH and H_2SO_4 to evaporate. After evaporation, 5 ml. of 6 N HCl were added, and the beaker was covered with a petri dish and heated 16 hours in the autoclave at 120° C. The liquid was then evaporated on a water bath, and the residue was neutralized and diluted to 200 ml. in a volumetric flask.

To determine the aminoid terminal residues, the microbiological assays were made on the samples blocked by the above procedure. Amino acid assay values showing decreases from the compositional values indicate aminoid termini of these amino acids.

Method for blocking the carboxoid terminal amino acid residues. In 50 ml. weighing bottles were placed aliquots of the lysozyme proteolyzates (equivalent to approximately 50 mgm. of lysozyme) and 0.5 ml. portions of acetic anhydride. The bottles were placed in a vacuum dessicator over

NaOH and H_2SO_4. Evaporation usually took place within 8 to 10 hours. To the dry residue were added 25 mgm. of NH_4SCN, 4.5 ml. of acetic anhydride, and 0.5 ml. of acetic acid. The bottles were closed and heated on the boiling water bath with frequent agitation for 30 minutes. After removal from the water bath, 0.5 ml. of water was added, and the liquid was evaporated in a vacuum desiccator. During these runs, samples frequently stood at this stage for several weeks in a covered box.

Hydrolysis was accomplished by adding 5 ml. of 6 N HCl and autoclaving for 16 hours at 120° C. The HCl was evaporated on a water bath, and the residues were neutralized with dilute NaOH and diluted to 200 ml.

To determine the carboxoid terminal residues, the microbiological assays were made on the samples blocked by the above procedure. Amino acid assay values showing decreases from the compositional values indicate carboxoid termini of these amino acids.

An attempt to resolve the question of the reliability of the quantitative values, obtained for the samples subjected to the carboxoid residue blocking treatment, was made by setting up another chymotrypsin experiment for the 240 hour period. To each of two aliquots of 21.6 mgm. of proteolyzed lysozyme was added 2.82 mgm. of DL-valylglycyl-DL-phenylalanine containing an amount of L-phenylalanine

equivalent to that in the lysozyme aliquot. One of these and an aliquot without the addition were treated exactly as described for the carboxoid blocking procedure. Likewise, the other aliquot and an aliquot without the additional tripeptide were treated exactly the same way except that after evaporation of the acetic anhydride-acetic acid the residue was treated with 2 ml. of 1:1 ammonia for 30 minutes and again evaporated *in vacuo*. Acid hydrolysis was performed for 16 hours using 10 ml. of 3 N HCl, instead of the 6 N acid.

Microbiological assays were made for phenylalanine. The results for the four samples appear in Table 9.

Table 9

Microbiological Recovery of Phenylalanine from Carboxoid Blocked 240 Hour Chymotryptic Digest of Lysozyme

Treatment	Phenylalanine Recovery as Per Cent of Total Present
Carboxoid blocking	33
Carboxoid blocking with added tripeptide	37
Carboxoid blocking plus ammonia	33
Carboxoid blocking plus ammonia with added tripeptide	35

From the results of this experiment it would appear most probable that all of the phenylalanine in the 240 hour chymotryptic digest is carboxoid terminal and that about one-third of the terminal carboxoid residues of phenyalanine is recoverable after blocking. There is still doubt for this view since only 7 per cent of phenylalanine was recovered from a sample of the tripeptide alone subjected to the carboxoid blocking and ammonia treatments. It is possible, however, that the presence of much peptide material reduces the effectiveness of the ammonia in increasing the blocking effect.

Compositional values. Five ml. aliquots were taken from each sample just prior to placing them in the incubator. To these samples were added 5 ml. of concentrated HCl, and they were autoclaved for 16 hours at 120° C. The HCl was evaporated on a water bath, and the residue taken up in a little water, neutralized, and diluted to 200 ml.

Microbiological assay values for the amino acids obtained on these samples were considered the compositional values. These values, calculated as numbers of residues per mole of lysozyme of 14,700 molecular weight, appear as compositional values in the tables showing recoveries of amino acids after terminal residue blocking during the proteolysis of lysozyme.

RESULTS

The results of the microbiological assays for a sample of lysozyme treated by the carboxoid blocking procedure appear in Table 9, along with an average of the compositional values obtained from the experimental solutions.

Tables 10 through 21 contain the results of the assays on the proteolyzates after the aminoid terminal and carboxoid terminal residue blocking treatments. Values are given for samples treated at 24, 48, 120, and 240 hours of incubation.

These values again are calculated as the number of residues recoverable per mole of lysozyme of a molecular weight of 14,700.

Table 10

Amino Acid Composition of Lysozyme After Carboxoid Residue Blocking Treatment

Amino Acid	Compositional Values as Numbers of Residues*	Sample 1		Sample 2	
		Per Cent	Number of Residues	Per Cent	Number of Residues
Arginine	11.2	13.0	11.0	12.8	10.8
Aspartic Acid	19.8	18.0	19.9	-	-
Glutamic Acid	4.0	4.2	4.2	4.2	4.2
Glycine	11.1	5.7	11.1	5.8	11.3
Histidine	1.0	0.9	0.9	0.9	0.9
Isoleucine	7.0	5.7	6.4	6.1	6.8
Leucine	7.0	5.3	5.9**	5.4	6.0**
Lysine	5.7	5.8	5.8	5.9	5.9
Methionine	2.1	1.7	1.7	2.1	2.1
Phenylalanine	3.0	3.0	2.7	3.2	2.9
Proline	2.0	1.5	1.9	1.5	1.9
Serine	9.6	6.6	9.2	6.7	9.4
Threonine	6.7	5.4	6.7	5.1	6.3
Tyrosine	3.0	3.3	2.7	3.5	2.8
Valine	6.1	4.5	5.7	4.8	6.0

* These values are the average of the values obtained on the six experimental solutions.

** Showing a decrease of one residue, thus indicating a carboxoid terminal residue.

Table 11

Number of Residues of Amino Acids Recovered Per Mole After Aminoid Terminal Blocking During Chymotryptic Hydrolysis of Lysozyme

Amino Acid	Compositional Values*	Digestion Time			
		24 Hours	48 Hours	120 Hours	240 Hours
Arginine	11.0	8.9	9.0	6.4	5.8
Aspartic Acid	19.5	17.7	18.1	18.2	17.2
Glutamic Acid	4.0	4.3	4.1	4.1	3.8
Glycine	11.6	10.6	10.4	10.2	11.5
Histidine	1.0	1.1	1.0	1.0	1.0
Isoleucine	7.1	5.8	6.3	6.1	6.1
Leucine	6.7	6.9	6.9	6.9	6.9
Lysine	5.8**	4.6	4.1	3.3	2.8
Methionine	1.9	2.1	2.0	2.0	2.0
Phenylalanine	3.0	2.9	3.1	3.1	3.0
Proline	2.1	2.0	1.8	2.0	2.1
Serine	9.4	8.1	7.8	4.9	5.0
Threonine	6.8	6.9	6.8	-	6.8
Tyrosine	2.9	3.0	2.8	3.0	2.9
Valine	6.2	5.0	4.8	3.9	4.3

* Values obtained on the chymotrypsin experimental solution after acid hydrolysis without blocking treatment.

** Lysine has been shown to be the aminoid terminal residue (34, 85) and would amount to five residues on the aminoid blocked sample (34).

Table 12

Number of Residues of Amino Acids Recovered Per Mole After Carboxoid Terminal Blocking During Chymotryptic Hydrolysis of Lysozyme

Amino Acid	Compositional Values*	Digestion Time			
		24 Hours	48 Hours	120 Hours	240 Hours
Arginine	11.0	10.6	10.5	6.4	6.3
Aspartic Acid	19.5	18.5	18.5	18.4	18.1
Glutamic Acid	4.0	4.2	4.4	4.1	3.9
Glycine	11.6	11.0	11.1	11.3	11.1
Histidine	1.0	1.0	1.0	0.9	0.8
Isoleucine	7.1	6.8	6.7	7.0	6.6
Leucine	6.7**	5.0	4.8	4.1	4.0
Lysine	5.8	6.3	5.8	5.1	5.1
Methionine	1.9	1.1	1.1	1.1	1.0
Phenylalanine	3.0	1.8	1.3	1.2	1.0
Proline	2.1	2.1	1.9	2.0	2.0
Serine	9.4	10.2	9.4	7.8	7.8
Threonine	6.8	7.3	6.8	7.1	6.9
Tyrosine	2.9	2.0	1.6	1.1	1.0
Valine	6.2	5.6	5.6	5.2	5.3

* Values obtained on the chymotrypsin experimental solution after acid hydrolysis without blocking treatment.

** Leucine has been shown to be the carboxoid terminal residue of lysozyme (94). A quantitatively blocked carboxoid treated sample would therefore yield 6.0 residues. The decrease is believed subject to a -35% error.

Table 13

Number of Residues of Amino Acids Recovered Per Mole After Aminoid Terminal Blocking During Proteolysis of Lysozyme by Papain (Initial pH 5)

Amino Acid	Compositional Values*	Digestion Time			
		24 Hours	48 Hours	120 Hours	240 Hours
Arginine	11.2	10.1	10.2	8.1	8.1
Aspartic Acid	19.9	19.9	18.8	19.0	18.0
Glutamic Acid	4.0	4.8	4.5	4.1	4.0
Glycine	11.6	10.6	10.7	11.9	11.5
Histidine	1.0	1.0	1.0	1.0	1.0
Isoleucine	7.1	6.3	6.1	6.8	6.8
Leucine	6.7	7.1	7.0	7.2	6.7
Lysine	5.8**	5.3	5.0	4.4	4.3
Methionine	1.9	2.1	2.1	2.3	1.9
Phenylalanine	3.0	2.9	2.7	3.0	3.0
Proline	2.1	1.7	1.7	2.2	2.1
Serine	9.4	8.4	8.3	8.4	8.4
Threonine	6.8	7.1	6.5	-	7.2
Tyrosine	2.9	3.0	2.8	2.9	3.1
Valine	6.2	5.8	5.8	5.2	5.8

* Values obtained on the Papain pH 5 experimental solution after acid hydrolysis without blocking treatment.

** Explanation is the same as for Table 11.

Table 14

Number of Residues of Amino Acids Recovered Per Mole After Carboxoid Terminal Blocking During Proteolysis of Lysozyme by Papain (Initial pH 5)

Amino Acid	Compositional Values*	Digestion Time			
		24 Hours	48 Hours	120 Hours	240 Hours
Arginine	11.2	10.9	10.4	9.0	8.8
Aspartic Acid	19.9	18.7	18.9	19.9	19.0
Glutamic Acid	4.0	4.6	4.3	4.0	4.2
Glycine	11.6	10.7	11.1	11.3	11.1
Histidine	1.0	1.0	1.0	1.0	1.0
Isoleucine	7.1	7.0	6.7	6.5	6.8
Leucine	6.7**	6.6	6.3	6.7	6.5
Lysine	5.8	5.8	6.3	5.5	5.1
Methionine	1.9	1.8	1.8	2.2	2.1
Phenylalanine	3.0	3.0	2.7	3.0	3.1
Proline	2.1	2.0	2.0	2.1	2.1
Serine	9.4	9.7	9.7	9.9	9.5
Threonine	6.8	7.2	6.8	7.2	6.9
Tyrosine	2.9	3.0	2.9	3.0	3.2
Valine	6.2	5.8	5.6	5.6	5.6

* Values obtained on the Papain pH 5 experimental solution after acid hydrolysis without blocking treatment.

** Explanation is the same as for Table 12.

Table 15

Number of Residues of Amino Acids Recovered Per Mole After Aminoid Terminal Blocking During Proteolysis of Lysozyme by Papain (Initial pH 7.5)

Amino Acid	Compositional Values*	Digestion Time			
		24 Hours	48 Hours	120 Hours	240 Hours
Arginine	11.4	9.6	8.9	8.2	9.0
Aspartic Acid	19.9	19.1	18.3	19.0	18.1
Glutamic Acid	4.2	4.6	4.4	4.0	4.2
Glycine	10.8	10.7	10.7	11.3	11.3
Histidine	1.0	1.0	1.0	1.0	1.0
Isoleucine	7.0	6.3	5.7	6.2	6.1
Leucine	7.1	6.8	7.0	7.2	6.6
Lysine	5.8**	5.0	5.0	4.0	3.7
Methionine	1.9	2.0	2.2	2.1	1.9
Phenylalanine	3.0	2.9	2.4	2.9	2.9
Proline	2.1	1.9	1.7	1.7	1.9
Serine	9.4	9.1	9.0	7.0	7.4
Threonine	6.8	6.9	6.2	7.2	7.2
Tyrosine	2.9	3.0	2.7	3.0	2.9
Valine	6.2	5.8	5.8	5.4	5.3

* Values obtained on the Papain pH 7.5 experimental solution after acid hydrolysis without blocking treatment.

** Explanation is the same as for Table 11.

Table 16

Number of Residues of Amino Acids Recovered Per Mole After Carboxoid Terminal Blocking During Proteolysis of Lysozyme by Papain (Initial pH 7.5)

Amino Acid	Compositional Values*	Digestion Time			
		24 Hours	48 Hours	120 Hours	240 Hours
Arginine	11.4	9.7	8.7	9.5	11.7
Aspartic Acid	19.9	18.1	18.3	18.7	19.0
Glutamic Acid	4.2	4.4	4.2	4.0	4.0
Glycine	10.8	10.6	11.1	10.8	10.8
Histidine	1.0	1.0	1.0	0.9	1.0
Isoleucine	7.0	6.7	6.5	6.4	6.4
Leucine	7.1**	6.2	6.1	6.0	5.8
Lysine	5.8	6.0	6.0	5.5	4.9
Methionine	1.9	2.0	2.1	1.9	2.1
Phenylalanine	3.0	2.9	2.7	2.9	2.9
Proline	2.1	2.2	2.0	1.9	2.2
Serine	9.4	9.8	10.2	8.1	7.8
Threonine	6.8	7.0	6.9	6.9	6.8
Tyrosine	2.9	2.8	2.9	2.8	2.9
Valine	6.2	5.5	5.7	5.7	5.7

* Values obtained on the Papain pH 7.5 experimental solution after acid hydrolysis without blocking treatment.

** Explanation is the same as for Table 12.

Table 17

Number of Residues of Amino Acids Recovered Per Mole After Aminoid Terminal Blocking During Proteolysis of Lysozyme by Papain (Initial pH 8.5)

Amino Acid	Compositional Values*	Digestion Time			
		24 Hours	48 Hours	120 Hours	240 Hours
Arginine	11.1	8.5	9.5	8.5	8.7
Aspartic Acid	19.9	17.7	18.3	18.7	17.7
Glutamic Acid	4.2	4.6	4.6	4.2	4.8
Glycine	11.2	10.7	11.1	11.3	10.6
Histidine	1.0	1.0	1.0	0.9	0.9
Isoleucine	6.6	6.3	6.2	6.1	6.2
Leucine	7.2	6.6	6.6	6.7	6.5
Lysine	5.8**	5.0	4.9	4.1	3.8
Methionine	2.1	2.0	2.2	2.0	2.1
Phenylalanine	3.0	2.9	2.7	2.9	2.9
Proline	2.0	1.9	1.8	1.8	1.7
Serine	9.7	8.4	7.6	6.9	6.6
Threonine	6.7	6.7	6.8	6.9	7.1
Tyrosine	3.0	2.8	3.0	2.9	2.8
Valine	6.0	5.7	5.5	5.5	5.2

* Values obtained on the Papain pH 8.5 experimental solution after acid hydrolysis without blocking treatment.

** Explanation the same as for Table 11.

Table 18

Number of Residues of Amino Acids Recovered Per Mole After Carboxoid Terminal Blocking During Proteolysis of Lysozyme by Papain (Initial pH 8.5)

Amino Acid	Compositional Values*	Digestion Time			
		24 Hours	48 Hours	120 Hours	240 Hours
Arginine	11.1	9.6	8.9	8.5	9.0
Aspartic Acid	19.9	17.7	17.7	18.2	17.8
Glutamic Acid	4.2	4.4	4.3	3.7	4.0
Glycine	11.2	10.6	11.3	11.0	10.6
Histidine	1.0	1.0	1.0	0.8	0.9
Isoleucine	6.6	6.6	6.6	6.4	6.4
Leucine	7.2**	5.7	6.1	5.5	5.5
Lysine	5.8	6.0	6.0	4.8	4.8
Methionine	2.1	1.9	1.9	1.9	2.0
Phenylalanine	3.0	2.7	2.6	2.8	2.8
Proline	2.0	1.9	1.8	2.0	2.0
Serine	9.7	9.5	9.7	7.8	7.4
Threonine	6.7	6.2	6.7	6.8	-
Tyrosine	3.0	2.8	2.8	2.6	2.7
Valine	6.0	5.5	5.6	5.5	5.5

* Values obtained on the Papain pH 8.5 experimental solution after acid hydrolysis without blocking treatment.

** Explanation as for Table 12.

Table 19

Number of Residues of Amino Acids Recovered Per Mole After Aminoid Terminal Blocking During Tryptic Hydrolysis of Lysozyme

Amino Acid	Compositional Values*	Digestion Time			
		24 Hours	48 Hours	120 Hours	240 Hours
Arginine	11.8	11.1	9.0	8.2	9.3
Aspartic Acid	19.8	17.9	19.9	19.0	18.8
Glutamic Acid	4.0	4.2	4.1	4.0	4.3
Glycine	10.6	10.8	11.1	11.1	10.8
Histidine	1.0	1.0	1.0	0.9	0.8
Isoleucine	7.1	6.5	6.4	6.5	6.5
Leucine	7.1	7.2	6.8	7.0	6.9
Lysine	5.8**	4.9	5.0	4.4	4.2
Methionine	2.0	2.1	2.0	1.9	1.9
Phenylalanine	3.0	2.9	2.6	3.0	2.9
Proline	1.9	1.9	1.9	2.2	2.0
Serine	9.5	9.8	9.7	7.8	8.1
Threonine	6.7	7.3	6.5	6.8	6.9
Tyrosine	3.0	2.9	2.8	3.0	3.1
Valine	6.0	5.7	5.3	5.4	5.4

* Values obtained on the Trypsin pH 8.5 experimental solution after acid hydrolysis without blocking treatment.

** Explanation is the same as for Table 11.

Table 20

Number of Residues of Amino Acids Recovered Per Mole After Carboxoid Terminal Blocking During Tryptic Hydrolysis of Lysozyme

Amino Acid	Compositional Values*	Digestion Time			
		24 Hours	48 Hours	120 Hours	240 Hours
Arginine	11.8	10.1	8.8	9.0	9.0
Aspartic Acid	19.8	19.6	20.5	18.7	19.5
Glutamic Acid	4.2	3.9	3.7	3.9	4.2
Glycine	10.6	10.6	11.1	11.3	10.6
Histidine	1.0	1.0	1.0	0.9	0.9
Isoleucine	7.1	7.0	7.1	7.0	7.1
Leucine	7.1**	6.5	6.1	6.7	5.9
Lysine	5.8	5.9	5.9	5.5	5.0
Methionine	2.0	2.1	2.0	1.8	1.9
Phenylalanine	3.0	2.7	2.6	2.8	2.6
Proline	1.9	2.1	2.1	2.0	2.2
Serine	9.5	10.4	10.3	8.8	8.9
Threonine	6.7	7.0	7.3	6.8	6.9
Tyrosine	3.0	2.8	2.9	2.8	2.5
Valine	6.0	6.0	5.8	5.9	5.9

* Values obtained on the Trypsin pH 8.5 experimental solution after acid hydrolysis without blocking treatment.

** Explanation the same as for Table 12.

Table 21

Number of Residues of Amino Acids Recovered Per Mole After Aminoid Terminal Blocking During Peptic Hydrolysis of Lysozyme

Amino Acid	Compositional Values*	Digestion Time				
		12 Hours	24 Hours	48 Hours	120 Hours	240 Hours
Arginine	10.8	10.4	9.9	9.0	8.7	9.4
Aspartic Acid	-	19.9	19.9	20.8	19.6	19.7
Glutamic Acid	-	-	-	-	-	-
Glycine	11.0	11.1	-	11.1	11.3	10.6
Histidine	0.9	1.0	1.0	1.0	-	0.9
Isoleucine	6.8	6.0	5.8	5.6	5.3	5.7
Leucine	7.0	7.0	6.9	6.4	6.5	6.4
Lysine	5.8**	5.2	5.2	5.1	5.3	5.2
Methionine	2.1	2.2	2.1	2.6	2.3	2.6
Phenylalanine	3.1	3.1	3.2	3.2	3.1	3.0
Proline	1.9	1.9	1.8	1.8	1.7	2.0
Serine	-	8.2	-	-	-	-
Threonine	-	6.6	6.8	6.9	-	6.6
Tyrosine	3.0	3.0	3.1	3.1	2.9	2.8
Valine	6.0	5.9	6.0	5.4	5.1	5.3

* Values obtained on the Pepsin experimental solution after acid hydrolysis without blocking treatment.

** Explanation is the same as for Table 11.

Table 22

Number of Residues of Amino Acids Recovered Per Mole After Carboxoid Terminal Blocking During Peptic Hydrolysis of Lysozyme

Amino Acid	Compositional Values*	Digestion Time				
		12 Hours	24 Hours	48 Hours	120 Hours	240 Hours
Arginine	10.8	10.6	9.2	9.1	9.2	8.5
Aspartic Acid	-	19.7	19.3	19.8	19.6	19.8
Glutamic Acid	-	-	-	-	-	-
Glycine	11.0	11.1	11.0	11.1	11.1	10.6
Histidine	0.9	0.9	0.9	0.9	0.9	0.9
Isoleucine	6.8	6.6	6.7	6.7	6.8	6.7
Leucine	7.0**	6.0	5.8	5.9	5.9	5.7
Lysine	5.8	5.8	5.8	6.2	5.6	6.1
Methionine	2.1	2.0	2.3	2.1	2.6	2.3
Phenylalanine	3.1	3.0	2.9	2.8	2.8	2.6
Proline	1.9	1.8	1.9	1.9	2.2	1.9
Serine	-	9.9	9.5	9.5	10.3	9.9
Threonine	-	6.6	6.4	6.6	6.7	6.3
Tyrosine	3.0	2.8	3.0	3.0	2.9	2.9
Valine	6.0	5.9	5.6	5.5	5.6	6.0

* Values obtained on the Pepsin experimental solution after acid hydrolysis without blocking treatment.

** Explanation is the same as for Table 11.

DISCUSSION

Carboxoid Residue Blocking Experiments

From the initial experiments on the ammonium thiocyanate-acetic anhydride reaction with peptides, it appeared that under proper conditions the carboxoid residue of dipeptides and tripeptides could be made microbiologically and chromatographically unavailable. A higher percentage of remaining amino acids could be recovered from tripeptides than from dipeptides.

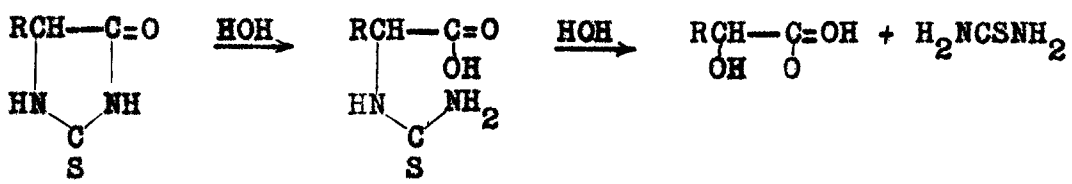
It is possible that diketopiperazines may be formed during the heating of dipeptides with acetic anhydride. This could prevent the blocking reaction from taking place quantitatively. At this time there does not seem to be a clear cut explanation for the effect of the ammonia on the ammonium thiocyanate-acetic anhydride treated peptides. According to Schlack and Kumpf (84) such treatment would cleave the peptide or amino acid from the thiohydantoin, but here decreased amounts of the remaining amino acids are recovered in some instances by a prolonged ammonia treatment.

The experiments involving various treatments of L-prolyl-L-leucine without subsequent acid hydrolysis in-

dicated that L. brevis can utilize the proline in the peptide form, but cannot use it in the acetylated peptide. Acetylation of the proline appeared to take place within four hours at room temperature (PL-1) in the presence of dioxane-HCl, and some blocking of the leucine took place. This appeared also to be true for one hour of treatment (PL-5) at room temperature.

From the fact that L. arabinosus can utilize to some extent the leucine in the form of the thiohydantoin, it would seem that in those cases after blocking treatment where the microbiological recovery of leucine is virtually nil there must be a degradation to another product.

It has been shown (51a) that proteins treated with ammonium thiocyanate-acetic anhydride followed by treatment with alkali contain considerable thiourea, which is probably a decomposition product of the thiohydantoin. This may be shown as follows:



If this is the reaction it would explain the low recoveries of leucine and valine in many of the runs in which these amino acids appeared as the terminal carboxoid residue. Hegstead (52) has shown that alpha-hydroxyisocaproic acid will not replace the requirement of leucine by

L. arabinosus, nor will alpha-hydroxyisovaleric acid replace valine for this organism. It is possible that the hydroxy acid cannot replace the amino acids for other microorganisms as well.

Further experiments on the blocking reaction showed that use of other acids besides acetic acid or anhydrous HCl reduced markedly the blocking of the carboxoid terminal amino acid residue.

Also, prior blocking of the free amino groups of the peptides either by benzoyl chloride or phenylisothiocyanate had no noticeable effect on the carboxoid blocking. In fact lower recoveries of aminoid residues were experienced in the benzoylated peptides because of the difficulty in hydrolyzing off the benzoyl moiety. A number of aminoid termini blocked by phenylisothiocyanate are not microbiologically recoverable (34, 41).

In the experiment in which no-ammonia, ammonia, and ammonia plus hydrogen peroxide treatments following ammonium thiocyanate-acetic anhydride reaction were compared it appeared that:

1. In the no-ammonia experiment the thiohydantoin did not appreciably decompose since the recovery of phenylalanine is what might be expected from a thiohydantoin.

2. By treating with ammonium hydroxide the recovery of the carboxoid residue, phenylalanine, was reduced

further, possibly due to decomposition of the thiohydantoin into thiourea and the hydroxy acid.

3. The presence of hydrogen peroxide in the ammonia in the third treatment reduced further the recovery of the carboxoid residue. In this case it appeared that further decomposition of the thiohydantoin had taken place (cf. 51a). Here some keto acid as well as hydroxy acid may be formed. Certain keto acids can replace the amino acids for microorganisms (52).

The use of phenyisothiocyanate in place of the ammonium thiocyanate in the blocking reaction did not seem promising.

The Dakin-West reaction, too, did not furnish evidence for use as a quantitative method for the carboxoid residues. A 90 minute heating caused almost complete destruction of leucine as well as valine in the dipeptide leucylvaline. A 60 minute heating caused the disappearance of valine, but as a quantitative determination was not made it is not known how much of the leucine, also, was destroyed. The method possibly has value as a qualitative method for small peptides, and it should not be ruled out altogether as a quantitative method or for use with larger peptides.

Initial Study of Extent of Enzymic Hydrolysis of Lysozyme

From the initial studies of the proteolysis of lysozyme it was seen that hydrolysis continued at a measurable

rate for at least 240 hours.

Since the absorbancy at 280 millimicrons of the peptides soluble in trichloroacetic acid is due to the presence of the aromatic amino acids or their residues, it is apparent that chymotrypsin produced many more trichloroacetic soluble peptides containing these amino acids than did the other enzymes. Trypsin produced a moderate number, but papain produced few soluble peptides containing the aromatic amino acids.

Proteolysis of Lysozyme

The reliability of the quantitative values determined for the aminoid blocked samples has previously been found (34, 41) to be as good as that for microbiological assays in general. Results as shown in Table 10 for carboxoid blocked unproteolyzed lysozyme would indicate a similar quantitative reliability for the carboxoid blocking method. However, the results of the recovery experiment in which valylglycylphenylalanine was added to the 240 hour chymotryptic hydrolyzate as shown in Table 9 would indicate about 65 per cent blocking of the carboxoid residues of phenylalanine.

Without further work, possibly involving the preparation and testing of the thiohydantoins of all the amino

acids, it cannot be stated definitely that the terminal carboxoid residues are 100 per cent or 65 per cent blocked. From results with synthetic peptides as well as lysozyme, it would appear that the amount of blocking of the carboxoid residues lies somewhere between these values.

Therefore, in computing the numbers of carboxoid terminal residues of an amino acid from the preceding tables, it seems more appropriate at this time to include a range. This range would be the difference between the assay values and the compositional values and 1.5 times this difference.

In discussing the results of the proteolytic studies, it seems simpler to speak of the average number of residues of an amino acid which appears to be terminal in one molecule of lysozyme of molecular weight 14,700* as shown in Tables 11 through 22.

Proteolysis by chymotrypsin

From Tables 11 and 12 it appears that chymotrypsin cleaves five bonds in which arginine is conjoined by its amino group and from five to seven in which arginine is linked through its carboxyl group. Two aminoid arginine linkages appear to be cleaved early in the hydrolysis. Some difficulty has been experienced in this assay so that

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* This meaning is employed throughout the discussion.

some reservation is held for the absolute values.

Aspartic acid has, also, given difficulty in assays, and this fact coupled with the large number of residues of this amino acid present in lysozyme would make a drop of two residues within the experimental error. It is, therefore, probable that none or very few of either aminoid or carboxoid residues of aspartic acid are opened by chymotrypsin.

Glutamic acid shows no aminoid or carboxoid linkages cleaved by chymotrypsin, and histidine shows only a very slight hydrolysis at the carboxoid linkage in 240 hours. Glycine assays are wanting in accuracy, also, but it appears possible from the values in Table 11 that one linkage involving the amino group of glycine is hydrolyzed.

Isoleucine shows cleavage of one aminoid bond in the first 24 hours but no further change.

Leucine shows no aminoid terminal residues even after 240 hours' hydrolysis. Two to three carboxoid terminal leucines are shown by the drop of two residues in the first 24 hours, but one has been shown to be the carboxoid terminal residue of the intact lysozyme molecule by Thompson (93) and is confirmed here (Table 10). A further drop of one residue takes place in 240 hours showing that one to one and one-half additional carboxoid termini of leucine have been hydrolyzed by chymotrypsin.

Lysine has been shown to be the terminal aminoid residue of the intact lysozyme molecule (34, 85) so that only a slight hydrolysis of the aminoid linkage is apparent in 24 hours. But in 240 hours an average of two residues have been freed at their aminoid end. Approximately one carboxoid terminal residue of lysine is found after 120 and 240 hours' hydrolysis.

Both methionine and proline show no cleavage of aminoid linkages during the entire period. However, one of the two methionines shows cleavage at the carboxoid terminus within 24 hours. Proline carboxoid residues cannot be determined by this blocking procedure (page 49).

The aromatic amino acids phenylalanine and tyrosine react identically to chymotryptic hydrolysis. No aminoid termini are found during the 240 hour period, but two of the three residues of each are hydrolyzed at their carboxoid linkage in 240 hours. From the results of recovery experiments involving phenylalanine (v.s. page 60) it would appear more probable that all three carboxoid linkages of each of the two aromatic amino acids have been cleaved. If this is true, it adds evidence to cleavage of one aminoid bond of glycine. Since the sequence tyrosylglycyl has been shown to exist in lysozyme (1), the hydrolysis of linkage involving the carboxyl group of tyrosine would expose a free amino group of glycine.

Approximately five linkages involving the aminoid group of serine are hydrolyzed in 240 hours while about two or three involving the carboxoid group are cleaved.

Threonine shows little hydrolysis of either linkage in 240 hours.

One peptide bond involving the aminoid group of valine is rapidly hydrolyzed, and about one more linkage is slowly hydrolyzed. An average of one carboxoid linkage of valine, per molecule of lysozyme, is slowly freed.

The hydrolysis of peptide bonds involving the carboxoid groups of phenylalanine, tyrosine, methionine, and arginine by chymotrypsin agrees with preferences found in synthetic peptide studies (v.s. pages 13, 14). The resistance to hydrolysis of bonds involving glutamic acid, glycine, leucine, lysine, histidine, serine, and threonine, in certain combinations, has been shown with synthetic substrates (v.s. page 15). Resistance to hydrolysis of linkages involving the "preferred" amino acids is seen in the present work with lysozyme, since many carboxoid and aminoid linkages involving them have not been hydrolyzed by chymotrypsin in 240 hours.

Hydrolysis by chymotrypsin of peptide bonds involving arginine and lysine has not been demonstrated previously. Since the specificity of trypsin involves the carboxoid linkages of these amino acids, it is possible that hydrolysis of these linkages is due to contamination of chymo-

trypsin by trypsin. However, it appears that hydrolysis of carboxoid linkages of arginine is even greater by chymotrypsin than with trypsin, so that contamination of chymotrypsin by trypsin is not an entirely satisfactory explanation.

Proteolysis by papain

Papain with an initial pH 5 shows no hydrolysis of either aminoid or carboxoid linkages of glutamic acid, glycine, histidine, leucine, methionine, phenylalanine, proline, threonine, or tyrosine as Tables 13 and 14 indicate. Calculations from these tables show that three aminoid and two to three carboxoid termini of arginine are cleaved, and few, if any, aspartic acid linkages are hydrolyzed.

It appears that nearly one aminoid bond and one carboxoid bond of lysine on the average per molecule of lysozyme are cleaved in 240 hours by papain. Slightly over one aminoid residue of serine is found to be terminal in 24 hours with no serine carboxoid residues terminal in 240 hours.

Papain at initial pH 7.5 differs from that at pH 5 in its effect on lysozyme in that two or three aminoid linkages of serine and two or three carboxoid linkages are hydrolyzed instead of one and none. An average of 0.7 residue of valine is hydrolyzed at its aminoid link.

At an initial pH 8.5 papain appears to hydrolyze be-

tween three and four serine aminoid bonds and between two and three carboxoid bonds. There is indication of minor attack on aminoid and carboxoid linkages of valine. Otherwise, the numbers of carboxoid and aminoid linkages hydrolyzed are the same as for the other initial pH values.

The specificities for papain-HCN as found in studies with synthetic substrates (v.s. pages 16 and 17) are borne out only to a minor extent in these experiments. The cleavage of linkages involving both the aminoid and carboxoid groups of lysine and arginine have been shown with synthetic peptides and derivatives. Perhaps most striking is the absence of hydrolysis of any glycine bonds in the present study, and only a few of the total number of bonds involving arginine and lysine are hydrolyzed.

The apparent effect of initial pH on the numbers of serine bonds hydrolyzed by papain-HCN may be partly due to inaccuracies in the microbiological assay, since frequent difficulties with this determination have been experienced. Also, long standing at higher pH values may cause splitting of the relatively labile bonds of serine.

Proteolysis by trypsin

Trypsin's preferences appear to be much like those of papain in that about one aminoid and one carboxoid linkages of lysine are hydrolyzed. Also, two to three aminoid and

two to three carboxoid linkages of arginine are cleaved. An average of about two serine aminoid and only one carboxoid linkages per molecule of lysozyme appear to be hydrolyzed. There is, also, evidence for slight attack on the carboxoid linkages of tyrosine and phenylalanine. Contamination of trypsin with chymotrypsin might be suspected from the latter observation, but no hydrolysis of carboxoid methionine is found here although it is found with chymotrypsin.

Specificities found for trypsin with synthetic substrates comprise the carboxoid linkages of the basic amino acids, arginine and lysine.

Proteolysis by pepsin

Microbiological assays on the peptic hydrolyzates in several instances are more equivocal than for the other preparations. It appears that only one isoleucine aminoid linkage is hydrolyzed. Two of the ten aminoid linkages of serine are hydrolyzed in the first 24 hours, and some attack on the aminoid linkage of valine is apparent in 120 hours. Lysozyme appears to be quite resistant to pepsin at pH 4, and no aminoid linkages of phenylalanine and tyrosine are attacked. Hydrolysis of the latter linkages would be expected from specificity studies with synthetic substrates. About two aminoid and two to three carboxoid linkages of

arginine are cleaved by the enzyme.

Alanine, cystine, and tryptophan have not been determined because of assay difficulties and destruction during treatments. From specificity studies tryptophan would be expected to react like the other aromatic amino acids with chymotrypsin, and cystine if linked with an aromatic amino acid would be expected to be hydrolyzed by pepsin at such a bond.

Bull and Currie (27) have claimed that pepsin reacting at pH 4 acts as a peptidase and will not hydrolyze protein, while at pH 1.8 it exhibits proteinase activity. Baker (6) has recently shown, however, that pepsin acts most rapidly at pH 1.8 on synthetic substrates composed of aromatic amino acids linked together. It would appear that the particular sequence of amino acids in a protein chain rather than the size of the molecule determines whether a proteolytic enzyme will hydrolyze or will not hydrolyze a given substrate at a given pH.

Most of the specificity studies of pepsin have been carried out at pH 4, and they have involved almost entirely such sequences as -glutamyltyrosyl-, -glutamylphenylalanyl-, -tyrosylcysteine, -cysteyltyrosine, and -cystyltyrosine. Following the above reasoning, it might be postulated that none of these particular sequences exist in lysozyme since no linkages of tyrosine, phenylalanine, or glutamic acid

were hydrolyzed. However, the contribution of adjacent amino acid residues to the preference of the enzyme for certain linkages is not known, so that such a conclusion cannot be drawn at this time.

It is evident that the sequences of amino acid residues in lysozyme and possibly the rigidity of the molecule due to cross linkages of cystine are contributing largely to the apparent preference of the four enzymes for particular linkages. This is born out by the facts that all of the "preferred" linkages as found for synthetic peptides are not hydrolyzed and that each enzyme hydrolyzes some arginine and serine bonds. In addition, chymotrypsin, trypsin, and papain split some lysine linkages.

The tripeptide arginylhistidyllysine and the dipeptides glycyltyrosine and tyrosylglycine have been isolated from partial acid hydrolyzates of lysozyme (1). Since no histidine bonds of lysozyme become terminal with any of the enzymes it is evident that the bonds arginyl-histidyl-lysyl are also resistant to hydrolysis by the four enzymes studied. Likewise, none of the enzymes hydrolyzes the glycyl-tyrosyl bond, and only chymotrypsin hydrolyzes the tyrosyl-glycyl bond.

In view of the apparent partial utilization of some thiohydantoins by microorganisms, another method of assay might be preferable to the microbiological method used here.

Even under the relatively severe conditions of the carboxoid blocking treatment, results in Tables 10 and 22 indicate that very little, if any, splitting of carboxoid linkages of serine or threonine occur from such treatment. Data in the several tables furnish evidence that only aminoid terminal amino acids with reactive side chains are blocked by the aminoid residue blocking procedure, along with the other aminoid terminal residues.

The considerable increase in pH values during, the proteolysis of lysozyme by papain and trypsin (Table 8), would seem to indicate that amide linkages as well as the aminoid linkages of the basic amino acids were being hydrolyzed.

CONCLUSIONS

1. The carboxoid residue blocking method as applied to lysozyme appears to reduce the microbiological availability of the carboxoid terminal residues by 65 to 100 per cent.

2. Preferences of chymotrypsin for peptide linkages of lysozyme do not disagree with specificities as found in synthetic substrates.

3. Preferences of papain for both aminoid and carboxoid linkages of lysine in lysozyme agree in a limited way with specificities as discovered from synthetic substrates.

4. As the initial pH of lysozyme solution is increased from 5 to 8.5, an increased number of serine bonds are hydrolyzed by papain.

5. The course of the proteolysis of lysozyme by papain and trypsin are similar.

6. Preferences of pepsin are conditioned by more than just a peptide bond involving an aminoid linkage of an aromatic amino acid.

7. Not all of the preferred (as indicated by synthetic substrate studies) linkages are hydrolyzed by papain, trypsin or pepsin.

8. All of the four proteases split bonds involving arginine, lysine, and serine in lysozyme.

9. Lysozyme is relatively resistant to proteolysis by pepsin at pH 4.

10. Chymotrypsin hydrolyzes lysozyme at pH 8.5 to a much greater extent than do the other enzymes under the conditions employed.

11. The aminoid terminal amino acid residue blocking method as applied here appears to be suitable for determining quantitatively the course of the proteolysis of aminoid linkages.

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