Sequential Degradation of Peptides from Their Carboxyl Termini with Ammonium Thiocyanate and Acetic Anhydride*

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ABSTRACT: Peptides react with ammonium thiocyanate and acetic anhydride under mild conditions to form peptidylthiohydantoins at their carboxyl termini. After separation from reagents by gel filtration, a peptidylthiohydantoin can be cleaved with acetohydroxamate, also under mild conditions, to a thiohydantoin characteristic of the carboxyl terminus and an acetylated peptide in which a new residue has become terminal. Since internal peptide bonds are not cleaved in either step, the degradation can be performed sequentially. Sequence has been determined by analyzing the peptide for its amino acid composition after each cycle of reactions. A number of model peptides derived from ribonuclease and insulin have been degraded through several stages each, with interpretable results. In the best case, six stages were possible, but, more typically, two or three stages of degradation can be performed successfully.

The method is limited in that carboxyl-terminal aspartic acid and proline are not removed, but all other residues, including asparagine, glutamine, and glutamic acid, do form acylthiohydantoins and are degraded. Degradations eventually become uninterpretable because the terminal residues are not removed completely each time. Removal is particularly poor when a residue precedes an aspartic or glutamic acid that is located three or more positions from the carboxyl terminus of the original peptide.

The conversion of acylamino acids into acylthiohydantions by ammonium thiocyanate and acetic anhydride was first described by Johnson and Nicolet in 1911. Since then, quite a few attempts have been made to apply this reaction, followed by a cleavage step, to the determination of carboxyl-terminal residues in peptides and proteins (Schlack and Kumpf, 1926; Fox *et al.*, 1955; Scoffone *et al.*, 1957; see Greenstein and Winitz (1961) for more references and a brief critical review). However, the *minimum* conditions for achieving complete reaction in the formation and cleavage of acylthiohydantoins have not been studied. As a consequence, the conditions used in the past were so vigorous that sequential degradation was impractical with all but the most stable peptides. Sequential degradation did seem to be a distinct possibility if substantially milder conditions for the cyclization and cleavage steps could be found. Such conditions now have been found and, together with several other improvements in procedure, they form the basis of the method described in this paper. The reactions involved are illustrated for a dipeptide in Figure 1.

Four principal improvements have been made. (1) The conversion of peptides into peptidylthiohydantoins requires excess acetic anhydride, in which most peptides are insoluble. The problem of insolubility can be circumvented by adding a mixture of ammonium thiocyanate, acetic acid, and acetic anhydride dropwise to a stirred solution of the peptide in 50% acetic acid. The charged and polar groups of the peptide are acetylated by the anhydride at the same time that water is removed, and no precipitation occurs. (2) A study of the rate and mechanism of the formation of peptidylthiohydantoins has led to the use of mild conditions for complete reaction. (3) Gel filtration on columns of Sephadex G-25 in 50% acetic acid has been used for separating peptidylthiohydantoins from excess reagents. Peptidylthiohydantoins with as few as three or four residues can be separated in this way. The high absorbancy of these compounds at about 280 mμ is of great use in locating them. (4) Cleavage of peptidylthiohydantoins with acetohydroxamic acid occurs under conditions mild enough so that amino acids that are not carboxyl terminal are not destroyed. The rate of cleavage can be followed spectrophotometrically, since thiohydantoins absorb maximally at about 260 mμ, about 20 mμ lower than acylthiohydantoins.

Materials and Methods

Reagent grade ammonium thiocyanate was recrystallized from absolute ethanol. After the hot solution had been filtered, it was cooled quickly to complete the crystallization and the product was immediately separated by vacuum filtration. Solutions that were cooled too slowly developed a distinct pink color. Acetic anhydride was redistilled through a 2-ft Vigreaux column; much better results were obtained with the distilled anhydride than with unpurified commercial reagent. It should be stored at −20° and distilled again after several months. Reagent grade acetic acid was used with-

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out purification. Pyridine was refluxed with excess phthalic anhydride, then redistilled through a Vigreaux column. Acetohydroxamic acid was synthesized according to Blatt (1944) and purified according to Wise and Brandt (1955). The white, crystalline product melted at 87–89°. Acetylthiohydantoins and thiohydantoins for model experiments were prepared according to Swan (1952a). The amino acids and peptides used for the experiments of Table XIII were commercial products. The peptides were chromatographically homogeneous and gave the expected amounts of amino acids after acid hydrolysis.

Peptides prepared by tryptic hydrolysis of bovine pancreatic ribonuclease A (Worthington) after oxidation with performic acid were separated as described by Hirs (1960) and by Smyth *et al.* (1963). Peptides derived from bovine insulin (Mann) were prepared from the glycyl and phenylalanyl chains after oxidation with performic acid or reduction and alkylation. Amino acid compositions of these peptides can be found in Tables I–XII.

Insulin was oxidized according to Sanger (1949). After desalting, the chains were separated on a 2 × 10 cm column of Dowex 1-X2 acetate. The phenylalanyl chain was eluted by 50 ml of a mixture of 162 ml of pyridine and 2.8 ml of acetic acid, made to 500 ml with water. The glycyl chain was eluted by 60 ml of 2 M sodium acetate in 50% acetic acid and precipitated from this solvent by the addition of acetone. Both peptides were desalted on Sephadex G-25 in 50% acetic acid. A peptide representing residues 1–16 was prepared by digesting 50 mg of oxidized phenylalanyl chain, dissolved in 10 ml of dilute ammonium acetate (pH 8) with 0.5 mg each of trypsin and chymotrypsin at room temperature for 6 hr, followed by desalting on a 2 × 50 cm column of Sephadex G-25 in 50% acetic acid. A peptide representing residues 1–22 was prepared similarly, but trypsin alone was used for the hydrolysis.

For reduction and alkylation, 20 mg of insulin was dissolved in 0.3 ml of a mixture of 1 g of guanidinium chloride, 6 mg of disodium EDTA, and 1.6 ml of 4 M Tris-HCl buffer (pH 8.6). The solution was flushed with nitrogen and 5 mg of dithiothreitol (Cleland, 1964) were added under nitrogen. The vessel was closed and, after 30 min at room temperature, 12 mg of recrystallized iodoacetamide was added. After 15 min more, 0.7 ml of 15% pyridine was added and the mixture of peptides was desalted on Sephadex G-25 in 15% pyridine. Reduced, carboxamidomethyl phenylalanyl chain was isolated in excellent yield by chromatography of the desalted peptides on a 2 × 20 cm column of DEAE Sephadex A-50, eluted with 25% pyridine, 0.4 M in formic acid. The glycyl chain was not recovered from the column, even when a similar solvent 2 M in formic acid was used.

Glucagon was obtained from the Eli Lilly Co. and separated from the accompanying lactose by gel filtration. Before sequential degradation, the peptide was modified with 2-hydroxy-5-nitrobenzyl bromide according to Barman and Koshland (1967), desalted by gel filtration, oxidized according to Sanger (1949), and desalted again.

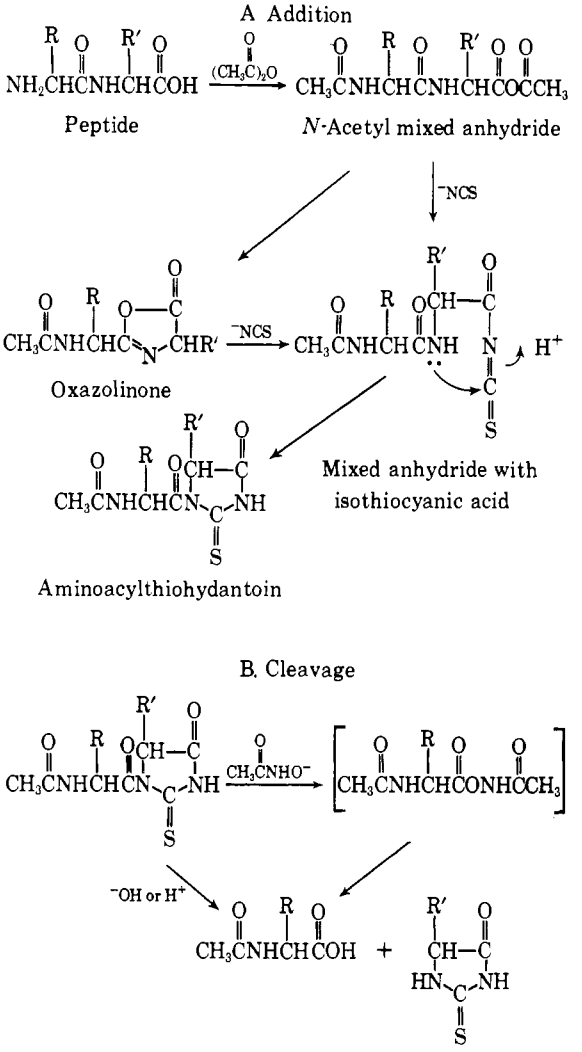


FIGURE 1: Degradation of a dipeptide from the carboxyl terminus.

Hydrolyses with 6 M HCl were carried out in evacuated, sealed tubes at 110°; a crystal of phenol was added to protect tyrosine. When carboxamidomethylcysteine was present, the tube was repeatedly flushed with prepurified nitrogen and reevacuated before it was sealed, to help prevent the destruction of this particularly labile amino acid. Amino acid analyses were performed with a Spinco Model 120B, modified for greater sensitivity with a long-path flow cell and range card (see Fruchter and Crestfield, 1965) so that 5–10 mμmoles of a single amino acid could be estimated accurately. Buffer flow rates were 100 ml/hr, with Spinco AA 15 and PA 35 resins in 58- and 10-cm columns, respectively. Because of the sensitivity of the analytical procedure, sequential degradations were usually run with a total of 0.2–0.5 μmole of peptide. With such small quantities, precautions were necessary to avoid adventitious contributions to the amino acid analyses from contaminated laboratory glassware. Any glassware that had been in contact with protein or peptide was rinsed with tap water, soaked overnight in dilute (about 0.2 M) sodium hydroxide, then rinsed again and washed in a glassware

TABLE I: Oxidized Phenylalanyl Chain, Insulin.[a,b]

Amino Acid	Theory	Molar Ratios of Amino Acids: Initial Peptide	Round 1	Round 2	Round 3
Lys	1	0.9	0.8	**0.2**	0.2
His	2	1.7	1.7	1.8	2.0
Arg	1	0.9	1.0	1.0	1.0
$CySO_3H$	2	1.8	1.8	1.9	2.0
Asp	1	1.0	1.0	0.9	1.0
Thr	1	1.0	0.9	0.9	0.9
Ser	1	0.9	0.9	1.0	1.0
Glu	3	*3.0*	*3.0*	*3.0*	*3.0*
Pro	1	1.0	0.8	0.9	**1.0**
Gly	3	2.9	3.2	3.2	3.0
Ala	2	1.9	**1.2**	1.0	1.1
Val	3	2.9	2.9	2.8	2.7
Leu	4	3.9	4.1	4.2	4.1
Tyr	2	1.9	1.9	2.0	1.9
Phe	3	2.9	2.9	2.9	2.7

[a] This legend is for Tables I–XII. Carboxyl-terminal degradations of peptides from ribonuclease, insulin, and glucagon. In each table, the number used as the basis of the calculation of molar ratios is italicized. The residue that should be removed in each round is in boldface type. The full procedure for degradation is designated method A; method B is a shortened procedure in which a portion of the peptidylthiohydantoin is hydrolyzed before cleavage and gel filtration after cleavage is omitted. See the Procedure section of the text for details. [b] Method B, NH_2Phe . . . Thr-Pro-Lys-Ala-CO_2H.

TABLE II: Reduced, Carboxamidomethyl Phenylalanyl Chain, Insulin.[a]

Amino Acid	Theory	Molar Ratios of Amino Acids: Initial Peptide	Round 1	Round 2	Round 3
Lys	1	0.9	1.1	**0.1**	0.1
His	2	1.8	1.8	1.9	1.8
Arg	1	0.9	1.0	1.0	0.9
CM-Cys	2	1.8	1.8	1.6	1.5
Asp	1	*1.0*	*1.0*	*1.0*	*1.0*
Thr	1	1.0	0.9	0.9	0.8
Ser	1	0.9	0.9	0.9	1.0
Glu	3	2.9	2.9	2.9	2.9
Pro	1	0.9	1.0	1.0	**0.7**
Gly	3	2.7	2.9	2.9	3.0
Ala	2	1.8	**1.0**	1.0	1.1
Val	3	2.9	2.9	2.7	2.7
Leu	4	3.7	3.8	3.7	Lost
Tyr	2	1.8	1.9	1.8	Lost
Phe	3	2.5	2.7	2.8	Lost

[a] Method B, NH_2Phe . . . Thr-Pro-Lys-AlaCO_2H.

washer. Blank analyses provided periodic assurance that the background level of contamination was acceptably low.

Procedure

Formation of Peptidylthiohydantoin. Dissolve the peptide in 0.5 ml or less of 50% acetic acid. Prepare a mixture of 100 mg of recrystallized ammonium thiocyanate, 4.0 ml of redistilled acetic anhydride, and 1.0 ml of glacial acetic acid. When the salt has dissolved, add all of the mixture slowly with swirling to the solution of peptide. Heat the mixture to 50° for 6 hr. Add 100 mg more of ammonium thiocyanate, swirl the liquid to dissolve the salt, and continue heating for 18 hr more at 50°.

Isolation of Peptidylthiohydantoin. Add 3 ml of water to the reaction mixture, swirl it, then allow the solution to stand for a few minutes to allow hydrolysis of the excess acetic anhydride to be completed. Transfer the solution to a 2 × 50 cm column of Sephadex G-25 (fine beads in 50% acetic acid). Deaeration of the eluent and swollen Sephadex with an aspirator prevents accumulation of gas pockets in the column. Wash the column with 50% acetic acid at any convenient flow rate. Collect 5-ml fractions and pool those with ultraviolet absorbancy. An inexpensive ultraviolet flow cell and recorder is a great convenience in locating the peptidylthiohydantoin. Yellow, probably polymeric, products of the decomposition of thiocyanate emerge from the Sephadex column ahead of the usual position of salt; peptidylthiohydantoins containing fewer than five or six residues merge with the beginning of this peak. Peptidylthiohydantoins larger than about seven residues are resolved. Although the first 20–25 ml of the peak does contain material that absorbs in the ultraviolet region, it contains very little salt so that, when small peptides are degraded, these fractions can be pooled and used in the cleavage step. Other yellow reaction products adsorb strongly to the Sephadex and require about two column volumes of wash to be eluted. The column is ready for use again when no more ultraviolet-absorbing material emerges.

Cleavage of the Peptidylthiohydantoin. Pool the peak tubes quantitatively and remove the solvent by evaporation in a vacuum. Redissolve the residue in 0.5 ml of 0.1 M acetohydroxamic acid, dissolved in 50% pyridine. (The reagent is stable at 4° for at least several months.) Heat the solution to 50° for 2 hr, then evaporate it to dryness. Redissolve the residue in 3 ml of 50% acetic acid, and desalt it again. Transfer a suitable portion of the solution for hydrolysis to a tube. The remaining peptide, after concentration, is used for the next round of degradation.

A somewhat shortened procedure (designated method B in Tables I–XII) may also be used. In this, the sample

TABLE III: Residues 1–22 of the Oxidized Phenylalanyl Chain, Insulin.[a]

Amino Acid	Theory	Molar Ratios of Amino Acids						
		Initial Peptide	Round 1	2	3	4	5	6
His	2	1.7	1.8	1.6	1.7	1.7	1.6	Lost
Arg	1	0.8	**0.2**	0.2	0.2	0.2	0.2	Lost
$CySO_3H$	2	1.9	1.9	2.0	1.9	**1.5**	1.4	1.4
Asp	1	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*
Ser	1	0.9	1.0	1.0	1.0	1.0	1.0	1.0
Glu	3	2.8	2.9	**2.4**	2.3	2.3	2.3	2.2
Gly	2	2.0	2.1	2.0	**1.6**	1.5	1.4	1.4
Ala	1	1.0	1.1	1.1	1.1	1.1	1.1	1.1
Val	3	2.7	2.9	3.0	2.9	2.8	**2.4**	2.1
Leu	4	2.7	3.9	3.9	3.8	3.8	3.8	**3.4**
Tyr	1	0.9	0.9	0.9	0.9	0.9	0.9	0.8
Phe	1	0.9	1.0	1.0	0.9	0.8	0.8	0.8

[a] Method B, NH_2Phe . . . Tyr-Leu-Val-$CySO_3H$-Gly-Glu-ArgCO_2H.

for analysis is removed immediately after the first desalting. The small amount of amino acid liberated from the thiohydantoin during acid hydrolysis does not contribute significantly to the analysis of the degraded peptide. Following cleavage and evaporation of the cleavage solvent, the peptide is redissolved in 0.5 ml of 50% acetic acid and the next addition reaction is begun without desalting again.

Hydrolysis and Analysis. Evaporate the portion to be hydrolyzed to dryness in a tube, then add 1 ml of 6 M hydrochloric acid and a small crystal of phenol. Seal the tube under vacuum and heat it to 110° for about 16 hr. The amount of peptide chosen for hydrolysis at each stage will, of course, depend upon the sensitivity of amino acid analysis. With the sensitive analyzer used in this work, one-tenth of the sample was hydrolyzed after each stage when 0.2–0.5 μmole of peptide was used in the degradation.

Results and Discussion

Degradation of Model Peptides. Twelve peptides have been degraded, with the results shown in Tables I–XII. Partial amino acid sequences for glucagon (Bromer *et al.*, 1957) and for peptides of insulin (Sanger, 1956) and oxidized ribonuclease (Smyth *et al.*, 1963) are indicated in each table. In most cases, the degradation can be interpreted for at least two rounds; the data of Tables IV and XI can be interpreted for six and four rounds, respectively. Many peptides were chosen especially because they contain acidic residues or amides at or near their carboxyl termini, since such residues are often troublesome and it seemed important to explore the limitations of the method carefully. In a more representative set of peptides, a greater fraction probably would have been degraded successfully for more than two rounds.

Removal of Carboxyl-Terminal Residues. Proline and aspartic acid are not degraded when they occur at the carboxyl terminus of a peptide. Furthermore, the free amino acids do not form thiohydantoins. In the case of proline, cyclization to form an acylthiohydantoin would require quarternarization of the imino nitrogen (see Figure 1) and hence does not occur.[1] Swan (1952b) has shown that both aspartic and glutamic acids form cyclic anhydrides readily when heated with acetic anhydride, and has claimed that these anhydrides do not react with ammonium thiocyanate to form acylthiohydantoins. In the case of aspartic acid, no spectrophotometric evidence for the formation of an acylthiohydantoin can be obtained under the conditions used in this paper; however, glutamic acid *does* give rise to an acylthiohydantoin (Table XIII), in partial contradiction of Swan's results. Furthermore, the glutamic acid in position 2 of the 22-residue peptide derived from the phenylalanyl chain of insulin is degraded (Table III). Difficulties in the degradation of carboxyl-terminal serine, threonine, lysine, and arginine have been reported in the literature (see Greenstein and Winitz, 1961), although reports of the synthesis of acylthiohydantoins or thiohydantoins corresponding to these amino acids have

[1] In Table II, and in a few other experiments with the phenylalanyl chain, some degradation of proline in round 3 did occur. In an experiment with the model tripeptide, glycylglycylproline, a product with the spectral characteristics of an acylthiohydantoin was formed in 37% yield (based on the extinction coefficient for acetylglycine thiohydantoin) upon heating a mixture of the peptide, acetic acid, acetic anhydride, and NH_4NCS to 100° for 1 hr, conditions much more vigorous than those ordinarily used. Turner and Schmerzler (1954) made a similar observation with a different small peptide and concluded that *N*-acylprolines are selectively cleaved under such conditions. Therefore, the acylthiohydantoin formed from glycylglycylproline is probably that of glycylglycine.

TABLE IV: Residues 1–16 of the Oxidized Phenylalanyl Chain, Insulin.[a]

Amino Acid	Theory	Molar Ratios of Amino Acids				
			Round			
		Initial Peptide	1	2	3	4
His	2	1.9	2.1	2.0	2.0	2.0
$CySO_3H$	1	1.1	1.1	1.1	1.2	1.2
Asp	1	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*
Ser	1	1.0	0.9	1.0	1.0	1.0
Glu	2	2.0	2.1	2.1	2.0	**1.9**
Gly	1	1.0	1.1	1.1	1.1	1.1
Ala	1	1.0	1.0	0.8	**0.6**	0.6
Val	2	1.9	2.0	1.9	1.9	2.0
Leu	3	2.9	3.0	**2.2**	2.1	2.2
Tyr	1	0.9	**0.1**	0	0	0
Phe	1	0.7	0.7	0.7	0.7	0.7

[a] Method B, NH_2Phe . . . Val-Glu-Ala-Leu-TyrCO_2H.

also been made (Swan, 1952a; Edward and Nielsen, 1953). Examples of the degradation of each of these residues when they are carboxyl terminal in a peptide may be found in Tables I–XII, indicating that the thiohydantoins do form.

Stability of Residues That Are Not Terminal. Amino acids that are neither terminal nor subterminal seem to be quite stable under the conditions of the cyclization and cleavage reactions. The hydroxyl groups of serine and threonine are undoubtedly acetylated during the first exposure to acetic anhydride, but apparently no β-elimination reaction ensues during cleavage. Histidine and carboxamidomethylcysteine may undergo very slow degradation, with loss of one-tenth residue or less per round; this has not obscured the results of any degradation in which these residues were present. No attempt has been made to degrade peptides containing cystine or cysteine. Methionine undergoes partial oxidation to methionine sulfoxide, which is not resolved from aspartic acid on the 55-cm column now most commonly used for analysis of neutral and acidic amino acids. This problem may be avoided by oxidizing methionine to methionine sulfone with performic acid before the degradation is begun. Tryptophan can be stabilized by reaction with 2-hydroxy-5-nitrobenzyl bromide (Barman and Koshland, 1967); if tryptophan and methionine are present in the same peptide, as in glucagon, the modification of tryptophan should precede oxidation, so that destruction of tryptophan and cleavage of the tryptophanyl peptide bond by performic acid is avoided (Table VI).

TABLE V: Oxidized Glycyl Chain, Insulin.[a]

Amino Acid	Theory	Molar Ratios of Amino Acids			
		Initial Peptide	Round		
			1	2	3
$CySO_3H$	4	4.0	4.2	**3.8**	3.6
Asp	2	2.2	**1.7**	1.5	1.4
Ser	2	1.8	1.9	1.9	1.9
Glu	4	*4.0*	*4.0*	*4.0*	*4.0*
Gly	1	1.1	1.0	1.1	1.1
Ala	1	1.1	1.0	1.0	1.1
Val[b]	2	1.7	1.6	1.6	1.6
Ile[b]	1	0.4	0.5	Lost	0.5
Leu	2	2.0	2.0	2.0	2.1
Tyr	2	1.8	1.8	1.7	**1.7**

[a] Method A, NH_2Gly . . . AspNH_2-Tyr-$CySO_3H$-Asp-NH_2COOH. [b] These residues are low because the sequence (IleVal) is incompletely hydrolyzed in 16 hr at 110°.

Incomplete Degradation and Precleavage. Usually, the initial round in the degradation of a peptide causes loss of nearly one residue of the terminal amino acid, but degradations eventually become uninterpretable because only partial removal of the terminal residue is obtained in subsequent rounds. This occurs even if cleavage is catalyzed by dilute alkali or concentrated HCl (see below), implying that incomplete cleavage of the preceding residue is not at fault. Side reactions during the formation of the peptidylthiohydantoin must be responsible, because prolonging the cyclization, raising the temperature, or adding more thiocyanate does not improve the yield. The nature of these side reactions is unknown.

The precleavage of subterminal residues, seen in a few of the degradations of Tables I–XII, probably occurs during the formation of a peptidylthiohydantoin from the preceding residue. An extreme example is given by α-aspartylalanine (Table XIII). When this

TABLE VI: Modified Glucagon.[a]

Amino Acid	Theory	Molar Ratios of Amino Acids					
		Initial Peptide	Round				
			A[b]	B[c]	1	2	3
Lys	1	1.0	0.9	1.1	1.0	1.0	1.0
His	1	0.9	0.9	0.9	0.8	0.7	0.6
Arg	2	2.0	2.0	2.2	2.1	2.0	1.9
Asp	4	4.1	3.7	3.9	3.5	**3.1**	2.6
Thr	3	2.9	2.7	2.7	**1.8**	1.8	1.8
Ser	4	3.6	3.4	3.4	3.3	3.2	3.1
Glu	3	3.0	2.9	2.9	2.7	2.6	2.6
Gly	1	1.0	1.0	1.0	1.0	1.0	1.1
Ala	1	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*
Val	1	0.9	0.9	0.9	0.9	0.8	0.8
Met or $MeSO_2$	1	0.9	0.8	0.9	0.7	0.7	**0.5**
Leu	2	1.8	1.8	1.9	1.7	1.6	1.4
Tyr	2	1.9	1.9	1.9	1.8	1.8	1.8
Phe	2	1.9	1.9	1.9	1.8	1.8	1.7
Trp	1		(39.8)[d]	(29.9)[d]	(19.8)[d]	(24.1)[d]	(26.2)[d]

[a] Method A, NH_2His . . . Tyr-Leu-Met-AspNH_2-ThrCO_2H. [b] Modified with 2-hydroxy-5-nitrobenzyl bromide; see the text. [c] Oxidized with performic acid after treatment A; see the text. [d] Molar absorbancy $\times 10^{-3}$ at 410 mμ. The molar extinction of modified tryptophan was determined after making a portion of the acid hydrolysate alkaline with NaOH, as described by Barman and Koshland (1967). The values above are appreciably higher than their reported extinction of 18,000, and there is quite a bit of variation, probably indicating that other components of the hydrolysate contribute to the absorbancy at 410 mμ.

TABLE VII: O-T-4, Ribonuclease.[a]

Amino Acid	Theory	Molar Ratios of Amino Acids				
			Round			
		Initial Peptide	1	2	3	4
Lys	1	1.0	**0.3**	0.1	0.2	0.2
His	1	1.0	1.0	0.9	0.8	0.7
$CySO_3H$	1	1.1	0.9	1.0	1.1	1.0
Asp	3	3.0	2.8	2.9	2.7	2.7
Thr	1	1.0	1.0	1.0	1.0	1.1
Ser	6	5.2	5.7	5.6	5.2	5.1
Glu	2	2.0	2.1	2.0	1.8	**1.7**
Ala	2	*2.0*	*2.0*	*2.0*	*2.0*	*2.0*
$MeSO_2$	3	2.9	2.8	**2.3**	**1.6**	1.3
Tyr	1	0.9	1.0	1.0	0.9	0.9

[a] Method A, NH_2GluNH_2 . . . GluNH_2-$MeSO_2$-$MeSO_2$-LysCO_2H.

peptide is subjected to the conditions of cyclization, only a product absorbing at 260 mμ (a thiohydantoin rather than an acylthiohydantoin) can be obtained, strongly implying that the β-carboxyl group of the aspartic acid catalyzes precleavage. Appreciable precleavage of serine occurs both in the degradation of O-T-7-9 (Table IX) and O-T-16 (Table XII). If substantial amounts of a peptide are available, it might be possible to overcome some of the limitations imposed by incomplete degradation and precleavage by purifying the peptide at an opportune time during the course of a degradation.

TABLE VIII: O-T-6, Ribonuclease.[a]

Amino Acid	Theory	Molar Ratios of Amino Acids: Initial Peptide	Round 1	Round 2	Round 3
Lys	1	1.1	**0.3**	0.2	*b*
Thr	1	1.0	1.0	0.9	0.9
Ser	2	1.8	1.7	**1.0**	**1.1**
Glu	1	*1.0*	*1.0*	*1.0*	*1.0*
Gly	1	1.0	1.2	1.0	1.0

[a] Method A, NH_2Glu-Thr-Gly-Ser-Ser-LysCO_2H. [b] Not analyzed.

TABLE X: O-T-8, Ribonuclease.[a]

Amino Acid	Theory	Molar Ratios of Amino Acids: Initial Peptide	Round 1	Round 2
Lys	1	0.9	**0.1**	0.2
Asp	1	1.0	0.9	**0.7**
Thr	2	1.8	1.5	1.5
Glu	1	*1.0*	*1.0*	*1.0*
Ala	1	1.0	1.0	1.0

[a] Method A, NH_2Thr-Thr-GluNH_2-Ala-AspNH_2-Lys-CO_2H.

The Effect of Acidic Residues or Amides on the Degradation. When aspartic acid or glutamic acid occur in positions other than the terminal or subterminal ones, degradation of the preceding residue seems to be poor. Such a situation occurs in Table IV (alanine in position 3 is degraded poorly and glutamic acid in position 4 is not degraded at all) and in Table XII (alanine in position 3 is degraded poorly; aspartic acid follows in position 4). However, in Tables III, VI, and IX, there are examples of the successful degradation of subterminal glutamic acid, asparagine, and glutamine.

If imides were to form from internal acidic residues during the course of the cyclization reaction, degradation of the preceding residue would be impaired, because its amino group would be acylated both by an α- and by a β- or γ-carboxyl group. It is already clear that formation of an imide does not compete well with formation of a thiohydantoin in the case of aspartylalanine (Table XIII). Several additional experiments were performed to test for the formation of imides, with the conclusion that, under the conditions of the cyclization reaction, imides do *not* form from internal asparagine or glutamine residues. No unambiguous conclusion could be drawn concerning the fate of internal aspartic or glutamic acid. A typical experiment was performed with O-T-7-9, which contains two residues of glutamine, one of asparagine, two of aspartic acid, and one of glutamic acid (four free carboxyl groups). The peptide was dissolved in 1 ml of a solution 2 M in guanidine hydrochloride and 1 M in taurine, pH about 4. To this solution was added 50 mg of *N*-ethyl-*N'*-3-dimethylaminopropylcarbodiimide hydrochloride (Hoare and Koshland, 1967). The resulting mixture was allowed to remain at room temperature overnight, then desalted on Sephadex G-25 in 50% acetic acid. Amino acid analysis revealed that 4.1 residues of taurine had been incorporated into the peptide, one for each free carboxyl group.[2] Another sample of O-T-7-9 was then exposed to the conditions of the cyclization reaction, except that NH_4NCS was omitted. After addition of water in the cold to destroy the excess acetic anhydride, the resulting solution was divided into three equal portions and each was evaporated to dryness at low temperature. Portion 1 was treated with water-soluble diimide and taurine, as described above. Portion 2 was cleaved with acetohydroxamate in pyridine, desalted, then treated with diimide and taurine. Portion 3 was exposed to 0.025 M NaOH for 5 min at room temperature, then neutralized with HCl and treated with diimide and taurine. The number of residues of taurine incor-

TABLE IX: O-T-7-9, Ribonuclease.[a]

Amino Acid	Theory	Molar Ratios of Amino Acids: Initial Peptide	Round 1	Round 2	Round 3
Lys	2	1.8	**0.9**	0.8	0.8
His	1	1.0	0.7	0.8	0.8
Arg	1	0.8	0.7	0.7	0.7
$CySO_3H$	2	1.5	1.3	1.4	1.3
Asp	3	3.2	2.9	2.8	2.8
Thr	1	1.0	1.0	1.0	1.1
Ser	2	2.2	2.3	1.9	**1.6**
Glu	3	3.3	3.1	**2.5**	2.5
Pro	1	1.0	1.0	1.0	1.2
Ala	2	2.2	2.2	2.2	2.1
Val	4	4.1	3.5	3.3	4.0
Leu	1	*1.0*	*1.0*	*1.0*	*1.0*
Tyr	1	1.1	0.9	1.0	1.1

[a] Method A, NH_2Asp . . . $CySO_3H$-Ser-GluNH_2-LysCO_2H.

[2] Taurine emerges about 20 ml before aspartic acid on the 55-cm column of the amino acid analyzer. In an experiment similar to the one described above, 12 residues of taurine were incorporated into oxidized ribonuclease A, which has 11 free carboxyl groups. This result indicates that modification of carboxyl groups with taurine is approximately quantitative, as is modification with aminomethanesulfonate (Hoare and Koshland, 1967).

TABLE XI: O-T-10, Ribonuclease.

Amino Acid	Theory	Molar Ratios of Amino Acids					
			Round				
		Initial Peptide	1	2	3	4	5
Lys	2	1.7	**0.8**	0.9	0.8	0.9	0.8
Thr	1	0.9	0.9	0.9	0.9	0.7	**0.6**
Glu	1	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*
Ala	3	2.8	2.7	**1.8**	**1.1**	**0.7**	0.7

[a] Method B, NH_2Lys-Glu-Thr-Ala-Ala-Ala-LysCO_2H.

porated into the peptide in each case was 3.9, 4.0, and 3.5, respectively. The result with portion 1 does not prove that imides involving aspartic or glutamic acid residues did not form, since hydrolytic cleavage may have occurred during exposure to the diimide or during the preceding work-up, with the regeneration of carboxyl groups; or the imides may have reacted directly with taurine. However, portions 2 and 3 were exposed to conditions that certainly would catalyze the cleavage of imides (see Bernhard *et al.*, 1962). If imides had been formed from glutamine or asparagine residues, cleavage would have resulted in the exposure of *new* free carboxyl groups. The results indicate that no new carboxyl groups were present and strongly suggest that asparagine and glutamine do not form imides under the conditions of the cyclization reaction.

In a similar experiment with O-T-8, which contains one asparagine, one glutamine, and the α-carboxyl group, only one residue of taurine was incorporated after cyclization without NH_4NCS and cleavage with acetohydroxamate, as would be expected if the glutamine and asparagine residues remained as the amides. The poor removal of subterminal asparagine in this peptide (Table X), a result obtained several times, is unexplained by imide formation.

Suppose that imides were to form slowly from internal aspartic and glutamic acid residues under the conditions of the cyclization reaction. If an aspartic or glutamic were subterminal, formation of an imide would not compete well with the formation of a thiohydantoin and the terminal residue would be degraded. If a residue of aspartic or glutamic acid were further in, an imide would have a chance to form slowly during the cyclization and would be cleaved during the subsequent exposure to acetohydroxamate, primarily to a β or γ peptide, rather than to the original α peptide (Smyth *et al.*, 1962). During the next exposure to acetic anhydride, the imide might well form again, but much more rapidly from the β or γ peptide than from the α peptide, with the result that thiohydantoin formation from the preceding amino acid would be blocked.

Rates of Formation of Acylthiohydantoins. The rates of reaction of several amino acids and peptides with NH_4NCS at 35° are given in Table XIII. The reactions do not follow pseudo-first-order kinetics because thiocyanate is unstable. Note that asparagine, cysteic acid, *S*-carboxymethylcysteine, acetylglutamic acid, serine, and arginine all do form acylthiohydantoins. Although small peptides react appreciably more slowly than amino acids, it is possible to find conditions under which extensive reaction occurs for each compound in the table, except aspartic acid and acetylaspartic anhydride.

To ensure that the cyclization reaction is complete, degradation of larger peptides is carried out at a higher temperature (50°) and a second addition of NH_4NCS is made to compensate for decomposition. The extent of cyclization under these conditions was checked, using the oxidized phenylalanyl chain of insulin, both by determining the absorbancy of the reaction product at 280 mμ (after separation on Sephadex) and by analyzing for the loss of carboxyl-terminal alanine, as in Table I.

TABLE XII: O-T-16, Ribonuclease.[a]

Amino Acid	Theory	Molar Ratios of Amino Acids			
		Initial Peptide	Round		
			1	2	3
His[b]	2	1.3	1.3	1.2	1.2
$CySO_3H$	1	Lost	0.8	1.0	0.9
Asp	2	*2.0*	*2.0*	*2.0*	*2.0*
Ser	1	1.0	0.5	**0.3**	0.3
Glu	1	1.1	1.2	1.1	1.2
Pro	2	2.0	2.0	2.0	2.2
Gly	1	0.9	1.0	1.0	1.0
Ala	2	1.8	1.9	1.6	**1.6**
Val[b]	4	3.4	**2.7**	2.7	2.7
Ile[b]	2	1.0	1.2	1.0	0.9
Tyr	1	0.9	1.0	1.0	1.0
Phe	1	0.9	0.9	0.9	0.9

[a] Method B, NH_2His . . . Asp-Ala-Ser-ValCO_2H. [b] These amino acids are low because the sequence (His-Ile-Ile-Val) is incompletely hydrolyzed in 16 hr at 110°.

TABLE XIII: Formation of Acylthiohydantoins from Amino Acids and Peptides.[a]

Compound	A_{280}, % of Maximum				
	1 hr	5 hr	24 hr	48 hr	96 hr
Alanine	40		77		
Alanine + NaOAc[b]	80	100			
Glycine + NaOAc[b]	45		100		
Asparagine	35		90	98	
Acetylaspartic anhydride		0			
Aspartic acid + NaOAc[b]			3		
Cysteic acid	50		87	88	
S-Carboxymethylcysteine	30		75	83	
Acetylglutamic acid + NaOAc[b]	34	82	100		
Serine + NaOAc[b]	61	95	100		
Arginine + NaOAc[b]	68	87	89		
Gly-Phe	14	38	70		
Ala-Lys	17	48	80		
Gly-Ser	13		50	70	70
Leu-Gly-Gly	9	21	52		
Leu-Gly-Gly + NaOAc[b]		50	75		
α-Asp-Ala[c]	18		67	71	86

[a] A solution of the compound in 0.5 ml of 50% acetic acid was mixed with 3.0 ml of acetic anhydride and 1.0 ml of a solution of NH_4NCS in glacial acetic acid (150 mg/ml). The mixture was kept at 35° and, at the times indicated, small portions were withdrawn and mixed with water. After about 0.5 hr (to allow the excess acetic anhydride to decompose), spectra were taken, using a sample without amino acid or peptide, but otherwise treated identically, as a blank. The extinction at 280 mμ was taken to be 1.75×10^4 for a 1-cm path. [b] Sodium acetate solution (1.0 ml), 150 mg/ml in glacial acetic acid, was added. [c] Maximum absorbancy was at 260 mμ.

Both methods indicated that the reaction was at least 95% complete.

Attempts to catalyze the reaction often gave different results with different compounds, complicating the choice of optimum conditions. For example, the general base, sodium acetate, markedly accelerates the reaction with amino acids (Table XIII) but, with large peptides, the rate of reaction with thiocyanate is significantly *slower* in the presence of sodium acetate.

When α-aspartylalanine is subjected to cyclization (Table XIII) the maximum absorbancy of the reaction product is at 260 mμ, indicating that the acylthiohydantoin is cleaved to a thiohydantoin, either during the reaction or upon dilution into water. Free thiohydantoins are not acetylated by acetic anhydride (Johnson and Nicolet (1911) and confirmed in this study), so that, if cleavage were to occur under the conditions of the addition reaction, the thiohydantoin would be stable. Cleavage in this case is probably catalyzed by the β-carboxyl group of aspartic acid.

On the Mechanism of Formation of Acylthiohydantoins (see Figure 1A). Johnson and Scott (1913) suggested that the mixed anhydride of isothiocyanic acid and the α-carboxyl of an acylamino acid was the immediate precursor of an acylthiohydantoin. This acyl isothiocyanate is formed by reaction of isothiocyanate anion with an activated carboxyl group, and there is clear evidence in the literature that the carboxyl group can be activated in a number of ways; for example, by formation of linear anhydrides (Swan, 1952c; Kenner *et al.*, 1953), *N*-carboxyanhydrides (Swan, 1952c), or oxazolinones (Johnson and Scott, 1913; Clarke *et al.*, 1949). Since oxazolinones are formed in acetic anhydride solution (Clarke *et al.*, 1949), probably from linear mixed anhydrides, it is not clear which of these is the precursor of the acyl isothiocyanate. In fact, it is highly probable that both function to some extent and it is certainly possible that the major reaction path may not be the same for different acylamino acids or peptides.

Many of the experiments of Table XIII were also carried out by pretreating the amino acid or peptide with acetic anhydride, to catalyze formation of oxazolinones first, and then adding thiocyanate. Although the acylthiohydantoins were initially formed faster in such experiments, the extent of reaction after 24 hr was always greater for samples that had not been pretreated. There is a strong implication from this result that acetic anhydride catalyzes reactions other than the formation of oxazolinones and it may be that such competing reactions are the cause of the incomplete removal of the terminal residue at each stage.

Formation of oxazolinones is stimulated by bases (see, for example, de Jersey *et al.*, 1966) and the cyclization of any acyl isothiocyanate might also be aided by a general base, which could help to remove a proton from the amide nitrogen. Since thiocyanic is a strong acid, with pK_a about 1 in water (Suzuki and Hagisawa, 1942), it is well ionized in acetic acid solution, and a

TABLE XIV: Deacylation of Acetylalaninethiohydantoin.[a]

Reagent (M)	Solvent	pH	Temp (°C)	$t/2$ (min)
Na_2CO_3 (0.05)	H_2O	11.5	25	15.5
Morpholine (0.5)	H_2O	10.8	25	85
Piperidine (1)	H_2O	12.4	27	0.8
Piperidine (1)	H_2O	11.1	25	3.0
Piperidine (1)	H_2O	10.7	25	9.7
Piperidine (1)	50% pyridine	11.1[b]	25	42
Imidazole (1)	H_2O	9.8	28	31.5
Imidazole (1)	H_2O	7.0	23	8.2
Imidazole (1)	4 M guanidine-HCl	7.0[c]	25	6.7
Hydroxylamine (0.05)	H_2O	6.1	24	1.3
Mercaptoethanol (0.05)	50% pyridine		25	>120
Acetohydroxamate (0.1)	H_2O	10.6	25	2.0
Acetohydroxamate (0.1)	H_2O	9.1	25	0.75
Acetohydroxamate (0.1)	H_2O	8.0	25	0.50
Acetohydroxamate (0.1)	H_2O	5.2	25	22.3
Acetohydroxamate (0.1)	50% pyridine		25	8
HCl (1)	H_2O		25	About 120
HCl (10)	H_2O		25	1.7
HCl (8)	Glacial acetic acid		25	3.3
HCl (1.9)	Anhydrous methanol		30	2.8
50% acetic acid	H_2O		25	Stable (45 min)
Anhydrous trifluoroacetic acid			25	Stable (30 min)

[a] Rates were measured spectrophotometrically. The pK_a of acetylalaninethiohydantoin is 6.9 at 25°. [b] pH measured in water before addition of pyridine. [c] An amount of acetic acid which would have given this pH when added to imidazole in H_2O was added to 1 M imidazole in 4 M guanidine-HCl.

general base probably would not help the nucleophilic attack of thiocyanate anion on the activated carboxyl very much. The effects of sodium acetate on the rate of formation of acylthiohydantoins from acylamino acids or peptides might well be explained if oxazolinone formation or cyclization of the acyl isothiocyanate were rate determining for the acylamino acids, but reaction with thiocyanate anion rate determining for peptides.

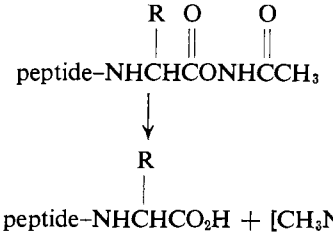
Finally, one can consider the close analogy between the formation of acylthiohydantoins from acetic anhydride and thiocyanate anion, on the one hand, and the formation of acylhydantoins from cyanate in acidic solution, on the other. In the latter case, the carboxyl group is activated by reaction with HNCO to form a mixed anhydride. The anhydride is then attacked by a cyanate anion to form the acyl isocyanate, which then cyclizes to the acylhydantoin (Stark, 1965). It is, in fact, possible to degrade the carboxyl-terminal alanine residue of the oxidized phenylalanyl chain of insulin with sodium cyanate, acetic acid, and acetic anhydride; however, the reaction requires a high concentration of cyanate and is much less convenient than a comparable experiment with thiocyanate because cyanate decomposes much more readily and because it exists mainly in the protonated form in acetic acid (pK_a of HNCO is about 4).

Cleavage of Acylthiohydantoins. Most of the experiments shown in Table XIV were done using acetylalaninethiohydantoin. The first group of reagents in the table are strong bases, and the rate of cleavage is roughly proportional to the pH in each case, suggesting that the attacking nucleophile is hydroxide ion and not the free base form of the reagent. Piperidine was used for cleavage in several degradations of the oxidized phenylalanyl chain of insulin, with good removal of the terminal residue at room temperature. Furthermore, a second stage of degradation could be carried out successfully. (This would not be expected if a peptidylpiperidine were formed during the first cleavage step.) Although there was no evidence for the cleavage of internal peptide bonds with piperidine, the recovery of threonine and serine was poor, probably because the high pH caused β elimination from *O*-acetylthreonine and *O*-acetylserine residues. For this reason, piperidine is not the reagent of choice for the cleavage.

The second group of reagents in Table XIV cleaves acetylalaninethiohydantoin by nucleophilic catalysis. The rates at a given pH are proportional to the first power of the concentration of nucleophile (data not shown in the table) showing that general base catalysis apparently is not important. Imidazole and mercaptoethanol are poor compared with hydroxylamine and ace-

tohydroxamic acid, indicating that anions of oxygen are highly favored for this cleavage. The pK_a for dissociation of the imide proton from acetylalaninethiohydantoin, determined spectrophotometrically, is 6.9 at 25°. The pH dependence of the imidazole-catalyzed cleavage shows that the uncharged acylthiohydantoin is more reactive than the anion. Similarly, in the acetohydroxamate-catalyzed cleavage, reaction between the anionic nucleophile and uncharged acylthiohydantoin is favored. In this case, the optimum pH for cleavage would be the average of the pK_a's of the reagent and the thiohydantoin. Since the pK_a of acetohydroxamic acid is about 9.4, the average is about 8.2; the data of Table XIV do indicate that the rate is indeed fastest at about pH 8.

Despite the fact that the reaction is rapid, hydroxylamine was not chosen for the cleavage of acylthiohydantoins. Jencks (1958) has shown that the initial attack on a carbonyl group is by the oxygen of hydroxylamine, and that the *O*-acylhydroxylamine that is formed initially is rapidly converted into the thermodynamically more stable hydroxamic acid by attack of the nitrogen of a second molecule of hydroxylamine. The hydroxamic acid would not be degraded since, for example, alaninehydroxamic acid does not form a thiohydantoin. When hydroxylamine is used for the cleavage of a large peptidylthiohydantoin, some of the new carboxyl-terminal residue must be present as the hydroxamic acid, but not all, since partial removal of the second residue can be obtained. However, the yields in round 2 are much better when acetohydroxamate is used. In this case, nucleophilic attack by the nitrogen cannot occur, and the product must have the structure, peptide–NHC(R)HC(=O)ONHC(=O)CH_3. It is not known whether this derivative is reactive enough to yield the acyl isothiocyanate directly with thiocyanate anion or whether it first decomposes to generate a free carboxyl group. The second possibility seems likely, since it is well known that *O*-acylhydroxamic acids undergo the Lossen rearrangement, but under milder conditions than hydroxamic acids (see, for example, Scott and Mote, 1927). In



any event, when cleavage is catalyzed by acetohydroxamate, the second round of degradation of large peptides proceeds with yields at least as good as those obtained when cleavage is catalyzed by HCl, which gives unambiguous formation of a free carboxyl group from the peptidylthiohydantoin.

The acid-catalyzed cleavages of acetylalaninethiohydantoin are summarized at the bottom of Table XIV. Somewhat surprisingly, neither anhydrous trifluoroacetic acid nor 70% aqueous trifluoroacetic acid catalyzes the reaction. Cleavage with methanolic HCl is very efficient but the methyl esters that are formed are not subject to subsequent degradation, making sequential degradation impossible. Although concentrated solutions of HCl are very efficient in removing the carboxyl-terminal residue, some nonspecific cleavage of peptide bonds probably occurs as well, judging from the changes in amino acid composition of peptides after several stages of degradation in which HCl was used for cleavage.

The cleavage by 10 M HCl of the peptidylthiohydantoin derived from the oxidized phenylalanyl chain of insulin occurs with a half-time of 26 min, in contrast to 1.7 min for the cleavage of acetylalaninethiohydantoin. Similar differences in rate between large peptides and small model compounds were encountered with the other reagents, including acetohydroxamic acid. For this reason, the conditions recommended for cleavage of peptidylthiohydantoin are much more severe than those that would suffice for cleaving compounds such as acetylalaninethiohydantoin. The cleavage reaction with acetohydroxamate is much slower in 50% pyridine than in water, but the former solvent must be used since many peptides are not soluble in water near pH 7, especially after their polar functional groups have been acetylated. All of the peptides in Tables I–XII dissolve readily in 50% pyridine.

The Nature of the Carboxyl-Terminal Fragment. Degradation of a peptide can be followed either by determining the amino acid composition after each round, as has been done in this paper, or by determining directly the fragment that is removed in the cleavage. The latter approach, essential if one hopes to degrade a protein, is complicated by the instability of thiohydantoins, which readily undergo hydrolysis of the five-membered ring to form thiohydantoic acids. This reaction has been studied in detail by Scoffone and Turco (1956), who found that hydrolysis occurs even in dilute alkali at room temperature. (The reaction can be followed spectrophotometrically since the wavelength of maximum absorbancy shifts from about 262 to about 237 mμ as hydrolysis proceeds.) Although free thiohydantoins are much more stable in dilute acetohydroxamate than in dilute NaOH, they are not recovered in good yield when a peptidylthiohydantoin is cleaved by the procedure recommended in this paper and then filtered on a column of Sephadex. Therefore, although thiohydantoins can be separated and determined directly (for example, by paper chromatography; Edward and Nielsen, 1953), such procedures will not be useful unless the thiohydantoins are stable during and after cleavage.

An alternative approach is to hydrolyze the mixture of thiohydantoin and thiohydantoic acid to an amino acid prior to quantitative analysis, as has been done for phenylthiohydantoins (Africa and Carpenter, 1966). Unfortunately, the hydrolysis of thiohydantoins in dilute alkali at 110° does not give good yields, even when the precautions specified by Africa and Carpenter are observed. Hydrolyses under much more extreme conditions result in moderate yields of some amino acids (Baptist and Bull, 1953) but are not likely to succeed with all because of decomposition. On the other hand, the hydrolysis of hydantoins or hydantoic acids in dilute

alkali gives excellent yields for every amino acid except threonine and serine (Stark and Smyth, 1963). Attempts to remove the sulfur from the thiohydantoin or thiohydantoic acid before hydrolysis, either oxidatively with H_2O_2 or reductively with Raney nickel, have given encouraging results that are being investigated further.

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