Determination of the Carboxyl Termini of Proteins with Ammonium Thiocyanate and Acetic Anhydride, with Direct Identification of the Thiohydantoins*

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ABSTRACT: The method described previously for sequential degradation of peptides has been extended to the determination of the carboxyl termini of proteins. Conversion of a protein into a proteinylthiohydantoin with ammonium thiocyanate and excess acetic anhydride can be carried out in homogeneous solution by using a mixture of hexafluoroacetone and acetic acid as the solvent. After separation from excess reagents by gel filtration, the proteinylthiohydantoin is cleaved in 12 M HCl.

The thiohydantoin derived from the carboxyl terminus is separated from modified protein by gel filtration and identified by thin-layer chromatography. In equivocal situations, it is possible to convert the thiohydantoin, after extraction from the thin-layer plate, into an amino acid in moderate yield by a procedure which combines alkaline hydrolysis and oxidation by H_2O_2. Two large peptides derived from insulin have been degraded successfully by this method for two and three cycles, respectively. The carboxyl-terminal residue has been identified unambiguously in bovine pancreatic ribonuclease A, sperm whale myoglobin, hens' egg white lysozyme, glucagon, and the catalytic and regulatory subunits of *Escherichia coli* aspartate transcarbamylase, but attempts to use the method sequentially with these proteins were not successful.

Determinations of the carboxyl termini of proteins with ammonium thiocyanate and acetic anhydride have been reported by several authors (see Greenstein and Winitz, 1961). Scoffone *et al.* (1957), using radioactive thiocyanate, have obtained quantitative data for ovalbumin, insulin, serum albumin, and lysozyme. In all of the previous applications, the conversion of a protein into a proteinylthiohydantoin was carried out in a heterogeneous reaction mixture under severe conditions. A principal feature of the work described in this paper is the description of mild conditions for carrying the reaction to completion in homogeneous solution, permitting attempts at sequential degradation to be made without disruption of internal peptide bonds. We have achieved sequential degradation, but only for peptides. However, it is possible that improvements in the details of the method, especially in the cleavage step, would allow sequential degradation of proteins as well. We do not plan to seek such improvements ourselves. For determination of the carboxyl-terminal residue of a protein, the procedure described below is a workable alternative to the well-known methods of hydrazinolysis and digestion with carboxypeptidases, and to the more recent procedures for selective labeling with tritium (Matsuo *et al.*, 1966; Holcomb *et al.*, 1968).

Materials and Methods

Recrystallized ammonium thiocyanate and redistilled acetic anhydride were prepared as described before (Stark, 1968). Hexafluoroacetone sesquihydrate, obtained from E. I. duPont de Nemours and Co., was converted into the trihydrate and redistilled through a Vigreaux column, essentially as described by Burkhardt and Wilcox (1967). Bovine pancreatic ribonuclease A and hens' egg white lysozyme were obtained from Worthington Biochemicals, bovine insulin from Mann Research, and glucagon from Eli Lilly and Co. Peptides derived from insulin were prepared as described before (Stark, 1968). The subunits of aspartate transcarbamylase were prepared according to Gerhart and Holoubek (1967). We thank Dr. Lubert Stryer for his generous gift of sperm whale myoglobin.

Crystalline thiohydantoins derived from lysine, asparagine, threonine, glutamine, glycine, alanine, valine, methionine, methionine sulfone, isoleucine, leucine, tyrosine, phenylalanine, and *S*-carboxamidomethylcysteine were synthesized by heating 10 mmoles of amino acid, 0.9 g of ammonium thiocyanate, 10 ml of acetic anhydride, and 1.3 ml of acetic acid to 100° for 30 min. After adding excess water, the solution was evaporated to yield a solid or an oil. The residue was redissolved in 12 M HCl for 30 min at room temperature, evaporated to dryness again, and recrystallized from water. The thiohydantoin derived from histidine was synthesized in the same way but recrystallized from 95% alcohol. The thiohydantoin derived from tryptophan was synthesized similarly, but the intermediate acetylthiohydantoin was precipitated in water and purified by crystallization from 95% alcohol before cleavage in 12 M HCl; tryptophan thiohydantoin was crystallized from ethyl acetate. Thiohydantoins derived from arginine, glutamic acid, and *S*-aminoethylcysteine were synthesized by dissolving the amino acid in a minimum volume of 50% acetic acid first, then adding the other reagents specified above, plus enough additional acetic

* From the Department of Biochemistry, Stanford University, School of Medicine, Stanford, California 94305. *Received July 22, 1969.* This investigation was supported by a grant from the National Science Foundation and by Public Health Service Research Grant GM11788 from the National Institute of General Medical Sciences.

† Supported by a research assistantship, Public Health Service Grant FR 05353.

TABLE I: Properties of Crystalline Thiohydantoins.

Amino Acid from Which Thiohydantoin Is Derived	Melting Points (°C)		Yields of Amino Acid After Alkaline Hydrolysis–Oxidation (%)[a]	
	Found	Lit.	No Chromatography	After Thin-Layer Chromatography
Tryptophan	190–192	190[b]	Destroyed	Destroyed
Lysine[c]	189–191		88	71
Histidine[d]	220 dec		33	16
Arginine[d]	148–150		84[e]	47[e]
Asparagine	252 dec	246[f]	72[g]	65[g]
Threonine[h]	264[i]	264[b,f]	Destroyed	Destroyed
Glutamic acid	115–116		64	44
Glutamine	189–191	190–191[f]	62[g]	28[g]
Glycine	229–231	227[f]	102	112
Alanine	165–166	163–165[b]	67	45
Valine	137–140	139–140[b]	62	53
Methionine	147–149	148–149[b]	42[j]	20[j]
Methionine sulfone	233–236		22	26
Isoleucine	132–133	131–133[b]	70[k]	40[j]
Leucine	177–178	172–173[f]	48	35
Tyrosine	206–208	211[b]	108	59
Phenylalanine	178–180	184–184.5[b]	87	65
S-Carboxamidomethylcysteine[l]	167–168		Destroyed	Destroyed
S-Aminoethylcysteine[c]	180 dec		Destroyed	Destroyed

[a] Corrected for recovery of valine hydantoin (internal standard); average of duplicate experiments. [b] Jackman *et al.* (1948). [c] The side-chain amino group is acetylated in the thiohydantoin. [d] Hydrochloride salt of the thiohydantoin. [e] Recovered as ornithine, which can be separated from lysine on the 55-cm column of the amino acid analyzer at pH 5.28. [f] Swan (1952). [g] Recovered as the acid. [h] The product is 5-ethylidene-2-thiohydantoin. *Anal.* Calcd: C, 42.24; H, 4.26. Found: C, 42.06; H, 4.33. [i] Rapid heating. [j] Recovered as methionine sulfone. [k] Isoleucine plus alloisoleucine. [l] *Anal.* Calcd: C, 32.86; H, 4.14; N, 19.14. Found: C, 33.07; H, 4.17; N, 18.64.

anhydride to react with the excess water. After cleavage in 12 M HCl, the thiohydantoin derived from arginine was purified by chromatography: the crude material was placed on a 2 × 10 cm column of Dowex 50-X8 in 0.2 M HCl and, after the column had been washed with several volumes of this solvent, the thiohydantoin was eluted with 100 ml of 3 M HCl. After removal of solvent in a vacuum, the product was recrystallized from methanol. The derivative of glutamic acid was purified after cleavage in 12 M HCl as follows: the crude material was placed on a 2 × 10 cm column of Dowex 1-X8 in 0.05 M HCl and the column was eluted with the same solvent. The thiohydantoin immediately followed a peak of yellow material which was not retarded by the resin; it was recrystallized from ethyl acetate–petroleum ether (bp 30–60°). The acetylthiohydantoin derived from *S*-aminoethylcysteine was cleaved in 0.01 M NaOH at room temperature for 30 min, then recrystallized from water after neutralization.

Properties of crystalline thiohydantoins derived from all of the amino acids commonly found in proteins are given in Table I, with the exception of aspartic acid and proline, which do not form thiohydantoins, and serine, from which a crystalline derivative was not obtained. Several commonly encountered derivatives of amino acids have been converted into thiohydantoins as well. Each crystalline thiohydantoin gave but a single spot upon thin-layer chromatography (Figure 1).

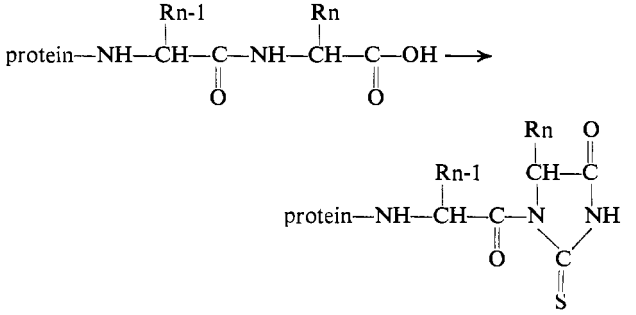
Procedure

The general plan of the procedure is illustrated in Scheme I.

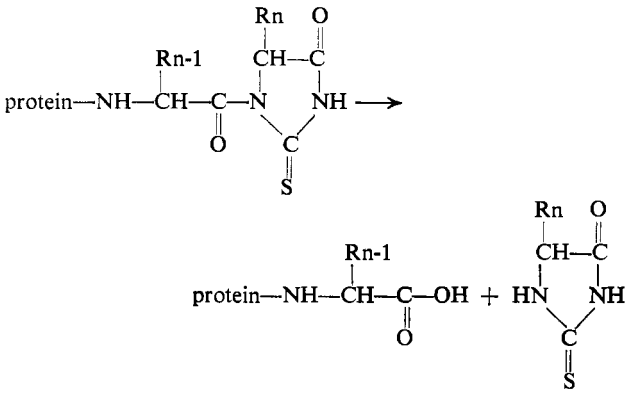
Formation and Isolation of Proteinylthiohydantoin. Dissolve about 1 μmole of protein in a mixture of 1.0 ml of hexafluoroacetone trihydrate and 0.35 ml of water. (Since 50 nmoles of thiohydantoin can be detected readily by the thin-layer chromatographic procedure described below, less protein can be used if material is scarce. We have not tried to determine the minimum amount of protein that can be used.) After solution is complete, add dropwise with swirling a homogeneous solution, prepared immediately before use, of 100 mg of ammonium thiocyanate in 1.0 ml of hexafluoroacetone trihydrate and 4.5 ml of acetic anhydride. Heat the mixture to 50° for 2 hr, add 100 mg more of ammonium thiocyanate, swirl the liquid to dissolve the salt, and continue to heat for about 18 hr more. Add 3 ml of water, wait a few minutes for the hydrolysis of excess acetic anhydride to be completed, then desalt the proteinyl thiohydantoin on a 2 × 50 cm column of Sephadex G-25, fine beads in 50% acetic acid, as described before (Stark, 1968).

SCHEME I: Steps in the Determination of the Carboxyl Terminus of a Protein with Ammonium Thiocyanate and Acetic Anhydride.

1. Formation of proteinylthiohydantoin



2. Separation from reagents on Sephadex G-25
3. Cleavage



4. Separation of protein and thiohydantoin on Sephadex G-25
5. Thin-layer chromatography of thiohydantoin
6. Conversion of thiohydantoin into an amino acid

Cleavage of the Proteinylthiohydantoin and Separation of the Products. Pool the peak tubes and remove the solvent by evaporation under vacuum. Redissolve the residues in 1 ml of 12 M HCl, allow the solution to stand at room temperature for 30 min, then remove the HCl rapidly with a good vacuum pump, without heating the sample above room temperature. Redissolve the residue in a small volume of 50% acetic acid and separate protein from thiohydantoin in the same solvent on a column of Sephadex G-25 identical with the one used for desalting. Remove the solvent from the thiohydantoin immediately, add a small volume of methanol, and proceed with thin-layer chromatography without delay.

Thin-Layer Chromatography. Plates precoated with silica gel and impregnated with fluorescent indicator (No. F-254, Brinkmann Instruments, Inc.) are used. Acetic acid should be refluxed with chromic acid and redistilled; all other solvents are Spectrograde. SYSTEM A: Activate the plate by heating it to 110° for 60 min just before use and develop it with a mixture of heptane (95 volumes), 1-butanol (65 volumes), and 99% formic acid (30 volumes). SYSTEM B: Do not activate the plate. Develop it with a mixture of chloroform (100 volumes), 95% ethanol (50 volumes), and glacial acetic acid (15 volumes). Spot the thiohydantoins onto the plates from methanol solution and run them along with appropriate standards. The spots are easily visible under ultraviolet light, since thiohydantoins quench fluorescence strongly; 50 nmoles of thiohydantoin can be detected readily.

Conversion of Thiohydantoins into Amino Acids. Remove solvent from the thin-layer plate under vacuum without delay scrape the spot from the plate into a small centrifuge tube, extract the thiohydantoin with methanol, pool the extracts (an internal standard may be added at this point if desired), and evaporate them to dryness in a heavy-walled hydrolysis tube. Flush the tube with a slow stream of nitrogen and, with nitrogen still blowing into the tube, add 0.2 ml of 0.2 M NaOH, previously degassed with nitrogen. Cover the tube with a small beaker as the nitrogen line is withdrawn and immediately place it in a boiling-water bath for 2 hr. Add 0.8 ml of water, then 10 μl of 30% H_2O_2 to the cooled tube. After 30 min at room temperature, evaporate the solution to dryness, add 0.4 ml of 1.5 M Na_2SO_3 and 0.4 ml of 2.0 M NaOH, seal the tube under vacuum, and heat it to 110° for 16 hr. Open the cooled tube, add 1.5 ml of 1 M HCl and evaporate the solution to dryness. Add 3.0 ml of water, stir, centrifuge the precipitated silicates, and analyze 1.0 ml of the supernatant solution.

TABLE II: Results of Carboxyl-Terminal Degradations with Peptides and Proteins of Known Structure.

Peptide or Protein	Residues Identified as Carboxyl Terminal
Oxidized phenylalanyl chain, bovine insulin[a]	Ala (I); Lys (II)
Tryptic peptide, residues 1–22 of the oxidized phenylalanyl chain[a]	Arg (I); Glu (II); Gly (III)
Bovine insulin[a]	Ala and $AspNH_2$
Bovine pancreatic ribonuclease A[b]	Val
Sperm whale myoglobin[c]	Gly
Hens' egg white lysozyme[d]	Leu
Porcine glucagon[e]	Thr
E. coli aspartate transcarbamylase	Leu
Catalytic subunit[f]	L
E. coli aspartate transcarbamylase	
Regulatory subunit[f]	$AspNH_2$

[a] Sanger (1956). [b] Smyth *et al.* (1963). [c] Edmundson (1965). [d] Canfield (1963). [e] Bromer *et al.* (1957). [f] Weber (1968).

Results and Discussion

Degradations of Known Compounds. The results of degradation of peptides and proteins of known structure or carboxyl-terminal sequence are shown in Table II. In each case, only a single spot appeared in the thin-layer chromatogram, in the position of the expected derivative. Degradation of the oxidized phenylalanyl chain of insulin was not attempted for more than two cycles because proline, which occurs three residues from the carboxyl terminus, is known to resist degradation by this procedure. (Aspartic acid is also not degraded; see Stark, 1968, for explanations.) In every other case, a cycle in addition to the one shown in the table was

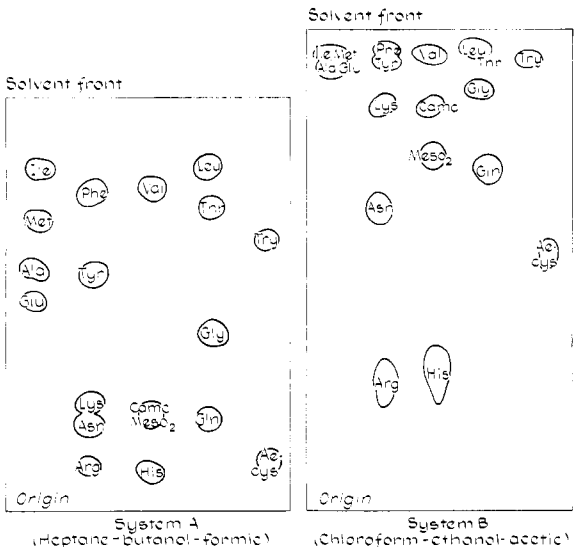


FIGURE 1: Thin-layer chromatography of standard thiohydantoins. Solvent systems A and B are described in the text. Each spot is labeled with the name of the parent amino acid. Abbreviations used: Camc, carboxamidomethylcysteine, $MeSO_2$, methionine sulfone; AECys, aminoethylcysteine.

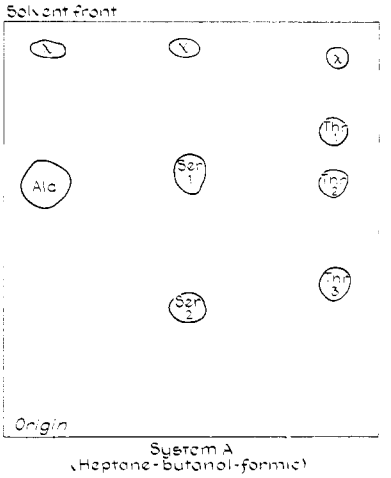


FIGURE 2: Thin-layer chromatography of reaction products of serine, threonine, and alanine. See the text for details.

attempted, but the evidence for the next residue was not clear-cut. Sometimes a faint spot appeared in the position appropriate for the second residue, but the usual result was either a faint diffuse smear or no spot at all. An intense spot was usually seen at the origin.

Formation of Proteinylthiohdyantoins. Every protein tested dissolved readily in hexafluoroacetone–water and remained soluble in the mixture with excess acetic anhydride and ammonium thiocyanate. The charged and polar groups of the protein are acetylated by acetic anhydride at the same time that excess water is removed. Therefore, it seems likely that solubility under these anhydrous conditions is a general property of proteins and that the conversion of a protein into a proteinylthiohydantoin can be carried out in a homogeneous solution in most instances. The extent of conversion of the carboxyl terminus of a protein into an acylthiohydantoin was checked in an experiment with ribonuclease A. The absorbancy of a solution of the proteinylthiohydantoin in 50% acetic acid was measured and the concentration of protein in the same solution was determined accurately by amino acid analysis. The absorbancy measured at 280 mμ was virtually identical with that calculated for complete reaction of the carboxyl terminus, assuming ϵ_M 1300 for each of the 3 tyrosine residues and ϵ_M 17,500 for the acylthiohydantoin. Haurowitz *et al.* (1957), using radioactive ammonium thiocyanate, had shown previously that one to two NCS residues per molecule were incorporated into insulin, ribonuclease, chymotrypsin, and lysozyme, consistent with a good yield of carboxyl-terminal thiohydantoin and little reaction elsewhere.

Cleavage of Proteinylthiohydantoins. HCl (12 M) was chosen as the reagent for cleavage because (1) it is an excellent solvent for proteins, (2) it does not destroy thiohydantoins, (3) it is volatile, and (4) reaction is rapid (Stark, 1968). Apparently, very little unspecific hydrolysis of peptide bonds occurs during brief treatment of a protein with 12 M HCl at room temperature, since many thiohydantoins would be expected in the second cycle if many new carboxyl termini were created during the first round cleavage. Cleavage was also attempted with dilute NaOH in water and with acetohydroxamate in 50% pyridine (Stark, 1968). Although 0.2 M NaOH was successful in a one-cycle degradation of insulin, several other proteinylthiohydantoins were insoluble in this reagent and could not be cleaved. Scoffone and Turco (1956) have previously described the instability of thiohydantoins in dilute alkali. Acetohydroxamate is difficult to use with proteins since the thiohydantoins are not readily separated from the excess reagent and substantial amounts of acetohydroxamate interfere with thin-layer chromatography. Judging from the rate at which their spectra change, thiohydantoins are destroyed slowly by acetohydroxamate in 50% pyridine also, with a half-life of about 4 hr at 50°.

Separation of Thiohydantoins. Thin-layer chromatography of the crystalline thiohydantoins of Table I in two solvent systems is shown in Figure 1. Most of the thiohydantoins can be identified unambiguously, and equivocal situations can be clarified by converting the thiohydantoin into an amino acid after extraction from the thin-layer plate (see below). Threonine, represented by 5-ethylidene-2-thiohydantoin in Figure 1, and serine, from which we were not able to obtain any crystalline thiohydantoin, give rise to multiple spots. In Figure 2 are shown the thin-layer chromatograms obtained from alanine, serine, and threonine after exposure to ammonium thiocyanate and acetic anhydride, then 12 M HCl. Alanine gives rise to the expected thiohydantoin plus an additional spot, marked "x" in Figure 2, which probably results from not removing excess thiocyanate before cleavage and which is present in all three samples. Serine gives two spots. The one marked "Ser-1" has λ_{max} 267 mμ and accounts for 22% of the maximum absorbancy expected; the one marked "Ser-2" has λ_{max} 265 mμ and accounts for 6% of the maximum absorbancy expected. (The recovery of absorbancy was 88% for alanine in a parallel experiment.) One of these two spots is probably 5-acetoxymethyl-2-thiohydantoin (*O*-acetylserine thiohydantoin); the other is probably *not* 5-methylene-2-thiohydantoin, which would be obtained by β elimination of the *O*-acetyl compound, since an exocyclic double bond would be expected to shift the spectrum of the

thiohydantoin about 60 mμ toward the red, as it does in the case of 5-ethylidene-2-thiohydantoin (see below). Threonine gives rise to three spots. The one marked "Thr-1," obtained in 9% yield, corresponds in chromatographic behavior and spectrum to authentic 5-ethylidene-2-thiohydantoin. Spot "Thr-2," obtained in 12% yield, has λ_{max} 267 mμ; spot "Thr-3," obtained in 3% yield, has λ_{max} 266 mμ. As with serine, one of these two spots probably represents the *O*-acetylthiohydantoin.

Procedures for identification of thiohydantoins alternative to thin-layer chromatography or paper chromatography (Edward and Nielsen, 1953) would certainly improve the method. In most attempts to do a second cycle of reactions with a protein, a peak with a spectrum appropriate to a thiohydantoin was obtained from the Sephadex column after cleavage, but thin-layer chromatography gave only an intense spot at the origin. We have attempted to volatilize thiohydantoins for gas–liquid partition chromatography with silylating reagents (see Pisano and Niall, 1969) or the methylating reagents diazomethane and dimethyl sulfate, but were frustrated by our finding that 2-thiohydantoins give rise to at least two major peaks, in contrast to 3-methyl-2-thiohydantoins, which give single derivatives (Pisano and Niall, 1969). One of the two peaks arising from methylation of 5-benzyl-2-thiohydantoin coincides with the position of authentic 5-benzyl-3-methyl-2-thiohydantoin; the nature of the second derivative is unknown. The thiohydantoins derived from alanine, valine, leucine, isoleucine, and methionine were volatile enough to be chromatographed without derivatization. We have not attempted to develop a system using liquid–liquid chromatography (Kirkland, 1969; Felton, 1969), but use of this new technique, which would circumvent the problem of converting thiohydantoins into volatile derivatives, seems especially promising. With such a system, cleavage with 12 M HCl may give results interpretable for several rounds, or perhaps other methods of cleavage such as acetohydroxamate in pyridine may become practical for proteins. Another advantage of liquid–liquid chromatography is that quantitation is more reliable than with thin-layer chromatography.

Extraction and Quantitation. Extraction of thiohydantoins from the thin-layer plates with methanol is virtually quantitative, judging from the recovery of absorbancy at about 262 mμ in the supernatant solutions. Most of the thiohydantoins absorb light maximally at about 262 mμ, with molar extinction coefficients close to 1.75×10^4. The exceptions are 5-ethylidene-2-thiohydantoin (from threonine) which, due to the exocyclic double bond, has λ_{max} 319 mμ and ϵ_M 2.5×10^4 and 5-(3-indolylmethyl)-2-thiohydantoin (from tryptophan) which, due to the indole ring, has a shoulder at 288 mμ, λ_{max} 266 mμ, and ϵ_M 2.15×10^4.

Conversion of Thiohydantoins into Amino Acids. Previous attempts to hydrolyze thiohydantoins to amino acids in NaOH were not successful (Stark, 1968), neither were our more recent attempts with 48% HBr (Turner and Schmerzler, 1954) or 1.2 M $Ba(OH)_2$ (Baptist and Bull, 1953). We have obtained yields of free amino acids of about 70% from valine and alanine thiohydantoins upon conversion into the thiohydantoic acids in dilute alkali (see below), followed by reductive desulfuration with acetone-treated Raney nickel for 15 min at room temperature in 0.4 M Na_3PO_4, then alkaline hydrolysis. Attempts to do the reduction more selectively at a lower pH were not successful. The best results in converting thiohydantoins into amino acids were obtained with a sequential procedure involving (1) hydrolysis to the thiohydantoic acid in dilute alkali (Scoffone and Turco, 1956), (2) treatment with alkaline hydrogen peroxide, and (3) alkaline hydrolysis. Except for those amino acids known to be destroyed by alkaline hydrolysis or hydrogen peroxide, the conversion of thiohydantoins into amino acids is achieved in good yield (Table I). For reasons that are not clear, the yields are not as good when the procedure is applied to the methanol extract of a spot from a thin-layer chromatogram after removal of the methanol under vacuum (Table I).

Haurowitz and Lisie (1950) had reported that thiourea could be quantitatively converted into urea by alkaline H_2O_2 and that the urea could be determined subsequently with urease. They obtained 0.2–0.5 mole of urea/mole of each of several thiohydantoins, implying that the α-carbon–nitrogen bond had been cleaved, since urease is highly specific for unsubstituted urea. In order to avoid this cleavage, we have used conditions milder than those described by Haurowitz and Lisie. Also, better yields are obtained if the thiohydantoins are first converted into thiohydantoic acids in alkali. It is well known that 5-alkyl-2-thiohydantoins can be oxidized readily by molecular oxygen in 0.5 M NaOH (see Edward and Nielsen, 1959, for a detailed discussion and description of the products) and we have found that exclusion of oxygen until conversion into the thiohydantoic acid is complete improves the yields significantly.

Stability of Thiohydantoins. Oxidation by molecular oxygen probably accounts also for our observation that exposure of a peptidylthiohydantoin to 0.05 M $NaHCO_3$ at room temperature causes the acylthiohydantoin spectrum to disappear without concomitant appearance of a thiohydantoin spectrum, with a half-time of about 3 hr. It is probably wise to minimize the exposure of peptidylthiohydantoins and thiohydantoins to air, since desulfuration by oxygen, analogous to the well-known desulfuration of phenylthiocarbamylamino acids (Ilse and Edman, 1963), may occur with these compounds, reducing the yields. Exposure to light should also be minimized.

Acknowledgment

We are grateful to Nanci Badger for her excellent technical assistance and to Dr. Bert Halpern for assistance with gas–liquid partition chromatography and associated techniques.

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Reaction of Tetranitromethane with Sulfhydryl Groups in Proteins*

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ABSTRACT: The oxidation of sulfhydryl groups with tetranitromethane has been examined using a series of model compounds. The reaction stoichiometry was determined colorimetrically by measuring the formation of nitroformate (ϵ_{350} 14,400) as well as by analysis of the other reaction products. Depending on the particular thiol studied and the molar excess of tetranitromethane employed, the course of the reaction leads mainly to a disulfide or to an acidic derivative. The acidic product has been identified as a sulfinic acid by chromatographic and electrophoretic analyses and by its conversion into a sulfonic acid on oxidation with iodine. The various factors which determine the relative amounts of the two reaction products are discussed. Oxidation of protein sulfhydryl groups with tetranitromethane results primarily in disulfide formation. No indication of interprotein chain peptide-bond formation mediated by tetranitromethane could be detected. Reaction of histidine, tryptophan, and methionine peptides with tetranitromethane was observed under certain conditions demonstrating the need for caution when using this reagent to modify proteins.

Nitration with tetranitromethane has proven to be a useful procedure for the investigation of tyrosyl residues of proteins (Riordan *et al.*, 1966; Sokolovsky *et al.*, 1966). However, the reagent is not totally selective for tyrosine. Specificity studies have shown that other residues are potentially reactive toward tetranitromethane (Sokolovsky *et al.*, 1966). In particular, sulfhydryl groups of model compounds such as, *e.g.*, glutathione, are oxidized readily to yield disulfides as the major product. Recently, it was found that tetranitromethane inactivates rabbit muscle aldoase by oxidation of thiol groups but disulfide-bond formation did not appear to represent the principal reaction pathway (Riordan and Christen, 1968). A number of reactions are known to modify thiol groups of aldolase and cause inactivation (Swenson and Boyer, 1957; Kowal *et al.*, 1965; Kobashi and Horecker, 1967). In instances where disulfide-bond formation is involved, reduction by mercaptoethanol generally leads to a complete restoration of activity (Kobashi and Horecker, 1967). However, exposure of tetranitromethane-inactivated aldolase to mercaptoethanol reactivated the enzyme only partially. The degree of reactivation seemed to be related to the extent of inactivation. Slightly inactive enzyme could be restored to virtually native activity but the enzyme with little residual activity could hardly be reactivated at all. Hence, some product other than a disulfide was thought to be formed. In order to elucidate the possible oxidative pathways, studies on the reaction of tetranitromethane with sulfhydryl groups in model compounds have now been carried out.

The conditions previously adopted to examine the specificity of the reagent toward other amino acid residues had been based on results with carboxypeptidase A (Riordan *et al.*, 1967). A fourfold molar excess of tetranitromethane was sufficient to modify one tyrosyl residue in this enzyme, which does not contain free thiol groups, with concomitant alterations of esterase and peptidase activities. Further, reaction of this tyrosyl residue was most favorable at pH 8.0 but the effect of pH on the specificity of the reagent was not examined

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