Analysis of Amino Acid Phenylthiohydantoins by Gas Chromatography

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SUMMARY

A gas chromatographic procedure has been developed for the quantitative analysis of submicrogram amounts of the phenylthiohydantoins of all the amino acids except arginine. The compounds are divided into three groups according to their volatility and need for derivatization (trimethylsilylation). The more volatile, relatively nonpolar thiohydantoins (Group I) are chromatographed with best results on columns coated with the stationary phase DC-560. For polar thiohydantoins of lower volatility (Group II), XE-60 is a much more suitable phase. The third group consists of thiohydantoins for which trimethylsilylation prior to analysis is either mandatory (aspartic, glutamic, and cysteic acids) or helpful (serine, S-carboxymethylcysteine, threonine, and lysine derivatives). A two column system employing the above stationary phases has been applied to the analysis of porcine thyrocalcitonin which was degraded by the three-stage form of the Edman procedure. In addition, a single column system has been developed utilizing a blend of three silicone phases which allows identification of all three groups of thiohydantoins in two 50-min runs (before and after trimethylsilylation).

Although the Edman procedure (1) for determining amino acid sequences of proteins and peptides has now assumed a classical role, there is still no entirely satisfactory way of identifying the 3-phenyl-2-thiohydantoin (PTH) derivatives of the amino acids removed in each successive step. Paper and thin layer chromatographic methods (2, 3) for direct identification of these compounds are cumbersome and quantitation at the submicrogram level is difficult. Hydrolysis of the derivatives to the free amino acids involves considerable labor and is not satisfactory for all amino acids even under carefully controlled conditions (4). Difficulties with these two major approaches have led many workers to use the "subtractive" method (5) in which the identity of the removed amino acid is ascertained by amino acid analysis of the residual peptide after each step. This is only practical with small peptides, since loss of a single amino acid residue from a protein or polypeptide is often difficult to measure with certainty. Loss of sample at each step, accumulation of blocked peptides, and the work and delay involved in repetitive amino acid analyses are other disadvantages. The use of the "dansyl" technique (6) in conjunction with Edman degradation is another indirect approach which is now widely used. After cleavage of the NH2-terminal amino acid by the Edman procedure, an aliquot of the shortened peptide is dansylated, hydrolyzed, and the new end group is identified as the dansyl-amino acid derivative by paper electrophoresis (7) or thin layer chromatography (8). This method has been used very successfully with short peptides in a number of laboratories; however, it cannot be readily quantitated and gives poor results with proteins.

Analysis of the phenylthiohydantoins formed at each successive step of the Edman procedure would seem a more direct and attractive approach to the problem, particularly in view of the recent successful automation of the Edman technique (9). An earlier investigation demonstrated that most amino acid phenylthiohydantoins could be successfully separated by gas chromatography (10); however, difficulties with the seryl, threonyl, asparaginyl, glutaminyl, lysyl, and arginyl derivatives limited the practical application of the procedure. These difficulties have been overcome by the use of new thermally stable and more polar silicone liquid phases and a new powerful silylating reagent which readily converts the less volatile and stable thiohydantoins to trimethylsilyl derivatives having excellent chromatographic properties. This study describes a gas chromatographic procedure for the analysis of the thiohydantoins of all the amino acids (except arginine) which are likely to be encountered in protein structural work. Although the procedure is undergoing further developments, it may be applied in its present form as described below for the 32-residue peptide hormone, porcine thyrocalcitonin (11). Finally, recent refinements in the procedure involving single column analyses are also described.

MATERIALS AND METHODS

Barber-Colman model 500 gas chromatographs equipped with hydrogen flame detectors, double column ovens with on-column injection ports, and temperature programmer were used. The injector temperature was at least the temperature of the column,
often 240° but never above 270°. The detector temperature was 280°, and the carrier gas was argon. In all chromatograms, full scale deflection was 3 x 10^-10 amps with a 5-mv recorder. Glass U or coiled columns employed throughout the study were silanized by treatment with 5% dichlorodimethylsilane in toluene (12). The columns were filled with this solution; after 10 min they were rinsed thoroughly, first with anhydrous methanol, then with acetone, and dried in air.

Gas Chrom P (100 to 120 mesh) was obtained from Applied Science Laboratories, Inc., State College, Pennsylvania. Chromosorb P and W (80 to 100 and 100 to 120 mesh) were purchased from Supelco, Inc., Bellefont, Pennsylvania. The supports were acid washed and silanized according to the procedure of Horning, VandenHeuvel, and Creech (12). Care was taken to prevent the further production of "fines" by avoiding excessive handling of the support. The silicone stationary phases, SE-30, OV-1, OV-22, OV-210, XE-60, DC-560 (formerly F-60), QF-1, and the silylating reagents, dichlorodimethylsilane, N,O-bis(trimethylsilyl)acetamide, and N,N-bis(trimethylsilyl)-trifluoroacetamide were purchased from Supelco, Inc. The organo-silicone phase ECNSS-S and polyester HI-EFF-3BP were obtained from Applied Science Laboratories, Inc.

The support was coated with different liquid phases according to the filtration procedure (12). The coating of mixed phase columns may be illustrated with reference to the "DXO" column. Ten grams of DC-560 and 3 g of XE-60 were separately dissolved in acetone, and each volume was made up to 100 ml. Three grams of OV-22 were dissolved in warm chloroform, and the volume also made up to 100 ml. A mixture was made of 56 ml of the "10%" DC-560 phase, 22 ml of the "3%" OV-22 phase, and 22 ml of "3%" OV-22 phase. This was used to coat the support by the above-mentioned filtration method. Columns were uniformly packed by gentle tapping of the walls, but not with an electric vibrator which could damage the support. Glass wool plugs used in the columns were silanized by dipping in dichlorodimethylsilane solution (5% in toluene), washing in methanol and acetone, and drying at 80° or on a Buchner funnel at room temperature.

Most amino acid phenylthiohydantoins were purchased from Mann Research Laboratories. Exceptions were the lysyl, tryptophan, histidyl, asparaginyl, glutaminyl, seryl, N-carboxymethylcysteiny1, and threonyl phenylthiohydantoins, which were synthesized by previously described procedures (13–16). The crystalline derivatives obtained gave sharp melting points at the expected temperatures. Seryl PTH and threonyl PTH were, in addition, shown to give single spots upon thin layer chromatography on silica gel, and had low ultraviolet absorption at 320 mµ, indicating the absence of significant amounts of the anhydroderivatives.

**Standard Solutions**—Standard solutions of the phenylthiohydantoins, except the asparaginyl, glutaminyl, histidyl, and cysteinyl acid derivatives, were made up as separate solutions in ethyl acetate at concentrations of 0.1 to 1.0 mg per ml. They were stored at 4° in screw cap vials fitted with Teflon cap liners. The derivatives of asparagine, glutamine, and histidine were dissolved in methanol (1.0 to 2.0 mg per ml) and stored similarly. The cysteic acid compound was kept as a 1.0 mg per ml solution in distilled water.

*Silylation*—Amino acid phenylthiohydantoin standards were reacted with N,O-bis(trimethylsilyl)acetamide (16) in conical glass reaction tubes having a capacity of 0.5 ml and an over-all length less than 5 cm. The tubes were sealed with Teflon-lined rubber septa, and samples were withdrawn with a 10-µl Hamilton syringe by piercing the septa. The needle can reach the tip of the tube permitting the handling of total volumes of only a few microliters. The septa were those commonly employed to seal gas chromatography columns (Supelco, Inc.); the Teflon tape was that used to line pipe threads (Chemplast, Inc., Wayne, New Jersey).

In a typical silylation, a 5- to 20-µl aliquot of a 1mg per ml solution of the amino acid phenylthiohydantoin standard in ethyl acetate was added to the tube. An equal volume of N,O-bis(trimethylsilyl)acetamide was added, and the vessel was immediately stopped to prevent access to atmospheric moisture. The contents were mixed vigorously (a mechanical vibrator is convenient) and allowed to incubate for 10 to 15 min in a 50° bath. Aliquots were injected directly into the gas chromatography column. For silylation of the asparaginyl, glutaminyl, histidyl, and cysteinyl acid derivatives, aliquots of the standard methanolic solutions were transferred to the reaction tube and evaporated to dryness under a stream of nitrogen. The dry residue was then redissolved in a small volume of pyridine or acetonitrile and treated with N,O-bis(trimethylsilyl)acetamide as described above for the other derivatives.

Silylation of samples obtained from Edman degradation were carried out similarly. The solvent used was ethyl acetate, except for the histidyl and cysteinyl acid derivatives. Samples suspected of containing these were dried and redissolved in pyridine or acetonitrile prior to silylation. Edman degradations were carried out by the three-stage method (18).

**Two Column System for Identification of Phenylthiohydantoins**—Gas chromatographic analysis of the Edman derivatives was performed with two columns. An all glass-coiled column 6 feet x 4 mm containing "10%" DC-560 was best used for Group 2. This designation refers to columns coated with mixtures of DC-560, XE-60, and OV-22. However, for non-group 2, N,O-bis(trimethylsilyl)acetamide was used routinely.

**Silica deposition**—A similar reaction tube may now be purchased from the Kontes Glass Company, Vineland, New Jersey, catalogue number K-749000. N,O-bis(trimethylsilyl) trifluoroacetamide is a new silylating reagent which is somewhat more volatile than N,O-bis(trimethylsilyl)acetamide (17). It decomposes in the flame ionization detector giving fluorinated compounds which reportedly (17) react with silica deposits in the detector, thus, retarding their accumulation. Silica deposits from N,O-bis(trimethylsilyl)acetamide, however, are easily blown out of the detector with an air jet or decomposed by injecting Freon TF (Supelco, Inc.) onto the column. Under the appropriate conditions, either reagent was suitable, but in the present study, N,O-bis(trimethylsilyl)acetamide was used routinely.
I and most of the Group III derivatives (Table I); while an all glass U column 2 feet × 2 mm containing "1%" XE-60 was used for Group II compounds and for distinguishing isoleucine from leucine. In a few instances, the recently developed single DXO column was also employed.

For convenience, detailed discussion of the manner in which the Edman degradation and the gas chromatographic system were used in conjunction will be presented under "Results" with data from actual degradations.

Quantitation—Yields of thiodyantoins were determined by comparing under identical conditions the peak areas of the unknowns with standards. Peak areas were measured by triangulation or disc integration. Adsorption of certain derivatives to the packing (see below) causes a departure from linearity at levels below 1 µg of derivative and necessitates the use of a standard curve.

Amino acid analyses were performed on a Beckman 120 B analyzer modified to allow rapid elution analyses at high sensitivity (19). Subtractive analyses during Edman degradation were based on amino acid analyses of aliquots of peptide taken after dissolving the reaction mixture in coupling buffer at the beginning of the next step of the degradation (before addition of phenylisothiocyanate). These aliquots were lyophilized and acid hydrolyzed.

RESULTS

Selection of Stationary Phases and Grouping of Amino Acid Phenylthiohydantoins

In preliminary experiments, the gas chromatography of amino acid phenylthiohydantoin standards was performed with a series of individual stationary phases. It was found that the amino acid derivatives commonly encountered in the Edman procedure could be divided into three groups which differed markedly in volatility and compatibility with different types of liquid phases (Table I).

Group I derivatives chromatographed well on all phases tested, but separations were better on relatively nonpolar phases, such as SE-30 and OV-1. The best results were obtained on DC-560 ("7 to 10%" coating), which separated all members of the group except the leucyl and isoleucyl derivatives with virtually no tailing (Fig. 1). Resolution of Group I was poor with columns containing OV-22 or XE-60.

Separation of the isoleucyl and leucyl phenylthiohydantoins was most difficult. Partial resolution was obtained with a number of liquid phases, including XE-60, QF-1, ECNSS-S, H1-EFF-SBP, and OV-210. Most satisfactory separation was obtained using an OV-210 column.

Group II derivatives are much less volatile and eluted much later than Group I on all columns tested. Their higher polarity also caused greater tailing which was markedly reduced, although not abolished, with the more polar liquid phases, XE-60, OV-22, and OV-17; all three phases gave excellent resolution of this group of derivatives. Best results for Group II compounds were obtained with XE-60 (Fig. 2).

Group III phenylthiohydantoins gave the least satisfactory results. No peaks were obtained for the glutamyl, aspartyl, or cysteic acid derivatives. Two peaks were seen with seryl and threonyl compounds, and finally, the lysyl and S-carboxymethylcysteiny1 derivatives gave peak areas 15 and 40%, respectively, relative to an equal weight of alanyl phenylthiohydantoin. It is possible to overcome these difficulties by silylation of the Group III derivatives.

![Fig. 1. Gas chromatographic separation of amino acid phenylthiohydantoins. Conditions: column, 6 ft × 4 mm; "10%" DC-560 on 100 to 120 mesh Gas Chrom P; the column was conditioned 16 to 24 hours at 325º, and an argon flow of 300 ml per min; column temperature programmed as indicated; argon flow, 115 ml per min. Although this experiment was carried out in the usual way (with 3 × 10⁻⁹ amps and a 5-mv recorder), the full scale deflection is not shown in this figure. Sample sizes 1 µg each except 0.5 µg of the methionyl, phenylalanyl, and 8 µg of asparaginyl, glutaminyl, and histidyl PTHs. The threonyl PTH standard gave two peaks. The time of the predominant peak obtained from the Edman degradation is indicated; the second peak coelutes with glycyl PTH. Because of their similar elution times, it is not possible to obtain unambiguous identification of the (asparaginyl, phenylalanyl) or (lysyl, histidyl, tyrosyl) derivatives. Peaks cannot be obtained on this column with the glutamyl, aspartyl, and CySO₂H phenylthiohydantoins.](http://www.jbc.org/)

<table>
<thead>
<tr>
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<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
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<tbody>
<tr>
<td>Alanine</td>
<td>Asparagine</td>
<td>Aspartic acid</td>
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<tr>
<td>Glycine</td>
<td>Glutamine</td>
<td>S-carboxymethyl-cysteine</td>
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</tr>
<tr>
<td>Valine</td>
<td>Tyrosine</td>
<td>Cysteic acid</td>
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<td>Leucine</td>
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</tr>
<tr>
<td>Proline</td>
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<tr>
<td>Phenylalanine</td>
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Table I

Grouping of amino acid PTH derivatives according to gas chromatographic behavior

Group I amino acids are most volatile and generally give symmetrical peaks. Members of Group II are least volatile. Histidine, asparagine, and glutamine show the greatest tendency to adsorb to the column packing, giving tailing peaks, and low responses. Group III derivatives include those which must be silylated before analyses (asparagine, glutamic, and cysteic acids) and others which, when silylated, have significantly better chromatographic properties. Silylation of Group I and II derivatives is not obligatory but can provide useful confirmatory data.
Silylation of Amino Acid Phenylthiohydantoins

Trimethylsilyl-proton exchange reactions with the highly reactive silyl donor, N,O-bis(trimethylsilyl)acetamide, have been shown to proceed rapidly and quantitatively under mild conditions (10).

In the first application (20) of this reaction to amino acid phenylthiohydantoins, it was stated that trimethylsilyl substitution occurs on the thiohydantoin ring as well as in the expected positions of the side chains. This is now supported by the independent study of Harman, Patterson, and VandenHeuvel (21), who used combined gas chromatography and mass spectrometry to demonstrate the substitution of a trimethylsilyl group for the protons of the hydroxyl, carboxyl, and amide groups and of the imidazole, indole, and thiohydantoin rings. Our own additional findings suggest that the silylation of some amino acid thiohydantoins may result in the formation of additional products.

However, the Group III derivatives give a major product following silylation in ethyl acetate or ethylene dichloride. While the unstable silyl cysteic acid, S-carboxymethylcysteinyl, and lysyl compounds give smaller peak areas than the other members of the group, they are nevertheless significantly greater than the untreated phenylthiohydantoins (Fig. 3). The structural identity of the peaks obtained upon silylation of Group III derivatives remains to be determined.

Silylation of Group I and II phenylthiohydantoins, while reproducible and quantitative in most cases, is complicated by the fact that no single set of conditions is suitable for all derivatives.

Fig. 2. Separation of Group II derivatives. Conditions: column, 2 ft x 2 mm, "10%" XE-60 on 100 to 120 mesh Gas Chrom P; column temperature, 265°; argon flow, 50 ml per min; sample size, 5 µg of the asparaginyl, glutaminyl, and histidyl PTHs, 1 µg of the tyrotyl and tryptophanyl phenylthiohydantoins.

Fig. 3. Group III phenylthiohydantoins and silyl derivatives. Phenylthiohydantoin peaks from separate chromatograms are superimposed with dashed lines. Conditions were same as Fig. 1 with column temperatures as indicated; argon flow, 115 ml per min. Conditions of silylation: equal volumes of standard solutions and N,O-bis(trimethylsilyl)acetamide, incubated 15 min at 80°C. Sample sizes: A, 1 µg of aspartyl and glutamyl PTHs silylated, 4 µg of cysteic acid PTH silylated; B, 8 µg of lysyl PTH, 2 µg of lysyl PTH silylated; C, 4 µg of S-carboxymethylcysteinyl PTH, 2 µg of S-carboxymethylcysteinyl PTH silylated; D, 4 µg of seryl PTH, 2 µg of seryl PTH silylated; E, 4 µg of threonyl PTH, 2 µg of threonyl PTH silylated. TMSi, trimethylsilyl.
Polar solvents such as acetonitrile and pyridine, which are most effective in dissolving the crystalline histidyl, asparaginyl, and glutaminyl derivatives, favor the formation of additional reaction products when used with certain other amino acid thiodyantoins (e.g., glycy1 and tryptophanyl). In all solvents tried, using the standard incubation temperature of 50° C and various reaction times up to 1 hour, the asparaginyl and glutaminyl derivatives were unique in that, upon silylation, they gave rise to other significant components as well as a dominant peak.

It should be remembered, however, that Group I and II derivatives may be identified and quantitated by direct injection; the silylation reaction was used merely as an occasional means of confirming their identity. Since the formation of more than one silylation product did not interfere with this identification, it is possible to use the conditions for silylation described under “Materials and Methods” section.

Application of Dual Column System to Edman Degradations

The three-stage form of the phenylisothiocyanate degradation was combined with the gas chromatographic detection method in the following way. The anilinothiazolinone derivatives were converted to phenylthiohydantoins by incubation in 0.2 ml of 1.0N HCl for 10 min at 80° under nitrogen (22). This solution was extracted three times with 1.0 ml of ethyl acetate to remove the thiodyantoins of all the amino acids except arginine, histidine, and cysteic acid. These derivatives are insoluble in ethyl acetate, and, thus, remain in the aqueous phase. The combined ethyl acetate extracts were concentrated with a stream of nitrogen to a volume of less than 0.5 ml. This solution was transferred to the reaction vials described above, and evaporated to dryness. The residue was redissolved in a small measured volume of ethyl acetate extracts were concentrated with a stream of nitrogen to a volume of less than 0.5 ml. This solution was transferred to the reaction vials described above, and evaporated to dryness. The residue was redissolved in a small measured volume of ethyl acetate solution. The combined ethyl acetate extracts (3 ml) were evaporated to dryness with brief warming of the vial in a water bath at 40-50° C. The Teflon-lined septa sealing the vials prevent reagents by gel filtration employing Bio-Gel P-2, the modified hormone was subjected to 16 cycles of degradation.

The first aliquot is injected onto the DC-560 column which permits the identification of most derivatives (Fig. 1). The phenylalanyl, asparaginyl, lysyl, tyrosyl, and isoleucyl leucyl derivatives are determined with the XE-60 column which permits unambiguous assignments (Fig. 2).

Another aliquot of the sample is silylated and injected on the preferred column when (a) no peak is obtained with the first injection and the identification of the aspartyl or glutamyl compound is required (DC-560), (b) either the seryl or the S-carboxymethylseryl derivative has to be identified (DC-560), (c) the seryl and tyrosyl derivatives need to be distinguished (XE-60), (d) the better response obtained with the silylated derivatives of S-carboxymethylseryl, serine, threonine, and lysine is desirable (DC-560), and (e) confirmatory data is useful on any of the remaining amino acid phenylthiohydantoins.

This completes the examination of the organic phase from the conversion step of the Edman degradation. If no identification has been made, the aqueous phase is examined for the derivatives of histidine, arginine, and cysteic acid. When the hydrochloric acid solution of the histidyl derivative is dried in a vacuum, it cannot be extracted into an organic solvent, because it is present as the hydrochloride salt. Consequently, to an aliquot of the conversion solution an equal volume of 1.0M aqueous Na2HP04 solution is added. The histidyl derivative, converted into the free base, may then be extracted into ethyl acetate solution. The combined ethyl acetate extracts (3 ml) were evaporated to dryness with a nitrogen stream, and the residue was dissolved in an appropriately small volume of methanol for injection onto the XE-60 column. The arginyl derivative may be identified by the Sakaguchi reaction or by thin layer electrophoresis. A standard phenylthiohydantoin derivative of cysteic acid has been successfully detected by gas chromatography as its trimethylsilyl derivative; however, there has not yet been occasion to attempt this with samples from Edman degradation.

Application of Dual Column System to Edman Degradations of Reduced and Alkylated Porcine Thyrocalcitonin

Porcine thyrocalcitonin is a 32-residue peptide hormone whose structure has been recently elucidated and confirmed by synthesis (11, 23-26). The reported amino acid sequence of the reduced and alkylated hormone is evident from the peptide fragments which were submitted to Edman degradation (Fig. 4).

Analysis of First 16 Residues of Reduced and Alkylated Hormone

Porcine thyrocalcitonin (1.25 μmoles) was reduced with mercaptoethanol and alkylated with iodoacetic acid. After separation of reagents by gel filtration employing Bio-Gel P-2, the modified hormone was subjected to 16 cycles of degradation.
**TABLE II**

Edman degradation and gas chromatographic analysis of 1.25 μmoles of reduced and alkylated thyrocalcitonin

<table>
<thead>
<tr>
<th>Step</th>
<th>Amino acid</th>
<th>Thiohydantoin μmoles</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>S-Carboxymethylcysteine</td>
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</tr>
<tr>
<td>2</td>
<td>Serine</td>
<td>0.60</td>
</tr>
<tr>
<td>3</td>
<td>Asparagine</td>
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<td>5</td>
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<td>6</td>
<td>Threonine</td>
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<td>7</td>
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<tr>
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<td>Valine</td>
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</tr>
<tr>
<td>16</td>
<td>Leucine</td>
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</tbody>
</table>

Fig. 5. Identification of Residues 1 and 2 as S-carboxymethylcysteinyl (SCMC) PTH and seryl PTH. The lack of shoulder (see arrow) on Step 1 peak suggests S-carboxymethylcysteine; the shoulder on the Step 2 peak suggests serine. Silylation of the derivatives provided confirmation of these assignments (Fig. 3). Chromatography conditions were same as Fig. 1; column temperature, 190°C.

Repetitive yields of the phenylthiohydantoin derivatives are shown in Table II. If the yields of amino acid thiohydantoins known to be stable under the degradation conditions (e.g. Leu4, Vala, Leua, Ala11, Leu14) are compared, it can be seen that there is an approximately linear decline in yield, corresponding to a loss of about 5% of the material initially present at each cycle.

Some comments may be made on the behavior of particular amino acid residues.

**S-Carboxymethylcysteine (Steps 1 and 7) and Serine (Steps 6, 5, and 10)**—As noted above, the phenylthiohydantoin derivatives of these amino acids give peaks having the same retention time. Earlier studies on serine (10), showed its peak to be the anhydro derivative which apparently is produced almost quantitatively from seryl PTH upon injection. Presumably the S-carboxymethylcysteinyl derivative forms the same product. In addition to the main seryl peak, a smaller later eluting peak is seen, as indicated by the arrow in Fig. 5. S-Carboxymethylcysteinyl PTH does not give rise to this additional peak; however, this difference alone is inadequate to distinguish the two. This is accomplished by silylation as shown also in Fig. 3. It seems likely that the peak given by silylated S-carboxymethylcysteinyl PTH is a breakdown product. Silylated seryl PTH also gives this peak, but as a minor product (Fig. 3). The gas chromatograms obtained from direct injection of samples from the first two steps of the degradation are shown in Fig. 5. The sequence (S-carboxymethyl-Cys-Ser) was confirmed by the use of silylation.

The absolute yields of seryl and S-carboxymethylcysteinyl thiohydantoins are low (Table II), usually in the range of 20 to 40% of theoretical yield. It was also observed that these derivatives (especially seryl) rapidly deteriorate over a period of several hours, unlike the crystalline standards.

**Asparagine (Steps 3 and 15)**—Identification of the asparaginyl residue at Step 3 of this degradation is shown in Fig. 6. The small earlier eluting peak seen in both standard and sample injections presumably represents a breakdown product produced.
at the time of injection. During the degradation, the asparaginyl derivative undergoes at least partial deamidation. Therefore, quantitation of the asparaginyl residue requires an essay for aspartyl PTH (silyl derivative). The silyl derivatives of the asparaginyl and aspartyl phenylthiohydantoins do not separate, however, on the XE-60 and DXO columns. Therefore, after quantitating the asparaginyl PTH in the samples, it is silylated and the silyl aspartyl PTH present is determined by subtracting the area due to silyl asparaginyl PTH. The percentage of deamidation and the total yield of the asparaginyl residue are then known.8

Threonine (Step 6)—Threonyl phenylthiohydantoin undergoes extensive β elimination during the conversion step of the Edman procedure. When analyzed on columns containing DC-560, the smaller peak, coeluting with glycyl PTH, represents residual threonyl PTH; the larger peak, eluting just before prolyl PTH, is the anhydrothreonyl derivative. An example is given below with the cyanogen bromide fragment (Experiment 5). Yields, calculated as the sum of both peaks, are usually 70 to 90% of theoretical yield.

Alanine (Step 11)—Fig. 7 shows the chromatogram obtained with the sample from the 11th degradation cycle. The major peak in the sample has the same elution time as alanyl PTH, which it clearly represents. The small peak eluting at 2 min is also seen when diphenylthiourea is injected. It is well separated from all the phenylthiohydantoins and does not interfere with identification or quantitation. The small peak emerging at 8 min immediately after the alanyl peak represents a small amount of the seryl derivative. The peaks seen at 11 min and 16 min represent small amounts of valyl PTH and leucyl PTH, respectively. Since Residues 8, 9, and 10 are valine, leucine, and serine, the appearance of these peaks probably indicates a small degree of incomplete reaction during this particular degradation.

Tyrosine (Step 12)—The reason for the low yield of tyrosine at this step is uncertain. Although losses of tyrosine during Edman degradation have been reported, this has been claimed not to occur when trifluoroacetic acid is used for cleavage (28) as it was in these experiments.

Tryptophan (Step 13)—The low yield of this phenylthiohydantoin is consistent with its known lability in anhydrous trifluoroacetic acid (18, 29).

Degradation on Middle Tryptic Peptide (T₆)

The yields of thiohydantoins are shown in Table III. The identification of the first 5 residues (Asn–Leu–Asn–Asp–Phe) presented no particular difficulty. After the sixth coupling, the reaction mixture was not extracted, but evaporated, to dryness. Cleavage was performed in the usual way. The trifluoroacetic acid was then evaporated, and the residue was subjected to the conditions of the conversion reaction. The solution would then be expected to contain the 6th residue as its phenylthiohydantoin derivative and the 7th residue and last as the free amino acid.

TABLE III

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<tr>
<th>Step</th>
<th>Amino acid</th>
<th>Thiohydantoin μmole</th>
<th>PTH μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Asparagine</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>Leucine</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>Asparagine</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>4</td>
<td>Asparagine</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>5</td>
<td>Phenylalanine</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>6</td>
<td>Histidine</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>7</td>
<td>Arginine</td>
<td>0.01*</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Free amino acid remaining after Step 6 was measured on the amino acid analyzer. The phenylthiohydantoin derivative of arginine cannot be determined by gas chromatography.

From the composition of the peptide and the specificity of tryptic cleavage the sequence could be presumed to be His–Arg. This was confirmed directly by extracting the histidyl derivative from one aliquot of the conversion solution, which had been first neutralized with 1 N Na₂HPO₄ as described above. The yield was measured by gas chromatography. Another aliquot was subjected to direct amino acid analysis; arginine was the only amino acid found. A third aliquot was acid hydrolyzed prior to amino acid analysis. Arginine and a small amount of histidine were found. The absence of aspartic acid, leucine, and phenylalanine indicated that the first 5 residues had been completely removed. The presence of a trace of histidine could be explained by incomplete coupling or cleavage at the 6th cycle, or to partial regeneration of free histidine from its thiodyantoin derivative during acid hydrolysis.

Degradation of COOH-terminal Tryptic Peptide (T₇)

Six cycles of degradation were performed. The yields of phenylthiohydantoins are shown in Table IV. Recovery of seryl PTH (Step 2) in low yield as expected. The lower yield of methionyl PTH cannot be explained at present. This methionyl residue in porcine thyrocalcitonin is known to be susceptible to oxidation to the sulfoxide form (30). However,
in the absence of information about the fate of methionine sulfoxide during Edman degradation, this suggestion must remain tentative.

Degradation on Cyanogen Bromide Fragment (BrCN)

The yields of phenylthiohydantoins are shown in Table V. Some reductions in the extent of extraction during this degradation were made to conserve peptide material. Glutamyl phenylthiohydantoin was identified as its silyl derivative. Identification of threonyl PTH at Step 6 is illustrated in Fig. 8. The difference in the ratio of the first to the second peak is due to the presence of more anhydrothreonyl PTH in the sample than in the recrystallized standard as noted above (Step 6). The peak seen at 20 min is a contaminant. The carboxyl-terminal prolinamide was identified by direct amino acid analysis of an aliquot of the residue after cleavage at the sixth cycle. Amino acid analysis of another aliquot after acid hydrolysis showed proline with only trace amounts of other amino acids. This subtractive analysis indicated that the coupling and cleavage steps had been almost quantitative.

These degradations provided the complete sequence of the thyrocalcitonin molecule. All the amino acids (except arginine) were identified and quantitated by gas chromatography. The molecule contains no lysine or isoleucine; however, identification of these residues has been accomplished during degradations on other peptides and proteins.

Single Column System

Although the two column system permits the analysis of all the thiohydantoins (except arginyl) encountered in sequence analysis, it requires multiple injections on separate columns, and it is most efficiently employed with two separate instruments. In order to simplify the procedure, a single column (the DKO column) which could be used for the identification of all three groups of thiohydantoins was developed. The stationary phases which were ultimately developed for this column consisted of a blend of 56 parts of “10%” DC-560, 22 parts of “3%” XE-60, and 22 parts of “3%” OV-22. On columns containing this blend, the prolyl derivative elutes with, or slightly earlier than, the glycyl compound. As the column became thoroughly conditioned with use, prolyl PTH moved to earlier retention times, ultimately eluting almost equidistant from the valyl and glycyl thiohydantoins. The column, once conditioned, remained stable for several months. Furthermore, this blend of phases is much more thermally stable than columns containing DC-560 and shows much less “bleed” at elevated temperatures than DC-560.

Chromatograms of the phenylthiohydantoins and the silylated Group III derivatives are shown in Figs. 9 and 10. A temperature programmed run of Groups I and II is seen in Fig. 9. The resolution of the Group I derivatives is good except for the leucyl and isoleucyl thiohydantoins.

The Group II derivatives are well resolved. Those phenylthiohydantoins of Group III which may be analyzed without silylation do not have unique retention times. The seryl and S-carboxymethylcysteinyl derivatives coincide with alanyl PTH, and the two peaks of threonyl PTH elute with the prolyl and glycyl compounds. Also, only marginal separation can be obtained between lysyl and tyrosyl PTHs. The phenylthiohydantoins were identified and quantitated by gas chromatography. The molecule contains no lysine or isoleucine; however, identification of these residues has been accomplished during degradations on other peptides and proteins.
Fig. 10. Group III phenylthiohydantoins and silyl derivatives. Phenylthiohydantoin peaks from separate chromatograms superimposed with dashed lines. Conditions were same as in Fig. 9 with column temperatures as indicated; argon flow, 90 ml per min. Conditions of silylation: equal volumes of standard solutions and N,O-bis(trimethylsilyl)acetamide incubated 15 min at 50°C. Sample sizes: A, 1 µg of aspartyl and glutamyl PTHs silylated, 4 µg of cysteic acid PTH silylated; B, 4 µg of S-carboxymethylcysteinyl PTH; C, 4 µg of seryl PTH, 2 µg of seryl PTH silylated; D, 4 µg of threonyl PTH, 2 µg of threonyl PTH silylated; E, 8 µg of lysyl PTH, 2 µg of lysyl PTH silylated. TMSi, trimethylsilyl.

dantoins of aspartic, glutamic, and cysteic acids must be silylated.

Fig. 10 shows the silylation technique applied to the Group III derivatives. Silylation of the seryl and S-carboxymethylcysteinyl derivatives separates them from each other and from the earliest eluting silyl compound, alanyl PTH. The silyl derivative of threonyl PTH gives a single peak distinct from the earlier eluting silyl glycyl and the unreactive prolyl thiohydantoins. A successful separation is also achieved upon silylation of lysyl and tyrosyl PTHs. The silyl PTH derivative of lysine elutes earlier and gives an improved response over its thiohydantoin. The silyl esters of aspartyl and glutamyl PTHs have unique retention times and good response while the silyl cysteic acid ester gives a poor response and coelutes with silyl S-carboxymethylcysteinyl PTH. This coelution, however, is not a problem since one or the other of these derivatives would have been chosen prior to the degradation. Injection, therefore, of an unknown phenylthiohydantoin onto the DXO column before and again after silylation would lead to a clear identification of all the naturally occurring amino acid derivatives except for the pair leucyl-isoleucyl PTH.10

DISCUSSION

The gas chromatographic procedure allows the determination of the removed amino acid at each successive step of the Edman degradation in contrast to the subtractive or dansyl procedures where analysis of an aliquot of the shortened peptide is required (to determine the missing residue or the new NH₂-terminal amino acid). Although these latter procedures are extensively employed for the analysis of peptides, they can be troublesome when applied to proteins or large peptides. The difficulty already inherent in measuring the loss of a single residue of a large molecule is complicated by the fact that blocked peptides from previous steps can accumulate in the sample. The dansyl procedure is known to be unreliable when applied to large molecules. Such problems do not arise when the cleaved residue is determined by direct analysis. Used in combination with the subtractive or dansyl methods, the gas chromatographic technique could serve to clarify ambiguities. In the analysis of thyrocalcitonin, the gas chromatographic technique provided the sole means of identification of the cleaved residue, but it should be apparent that the various approaches need not be mutually exclusive.

The data in Tables II through V proved most helpful in following the progress of the thyrocalcitonin degradation. Interference from contaminants was minimal and extraneous peaks having retention times similar to a thiohydantoin were not observed.

10 More recent work with the DXO column has shown nitrogen and especially helium to be superior carrier gases. The higher number of theoretical plates (helium 570, argon 425 per foot) allows the clear distinction of the isoleucine and leucine compounds. Retention times relative to the alanyl thiohydantoin (1.00) were isoleucyl 1.70, leucyl 1.72 with argon; and isoleucyl 1.74, leucyl 1.81 with helium.
TABLE V

<table>
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<tr>
<th>Step</th>
<th>Amino acid</th>
<th>Thiodyantoin pmol</th>
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<tbody>
<tr>
<td>1</td>
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<td>Phenylalanine</td>
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<td>Glycine</td>
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</tr>
<tr>
<td>6</td>
<td>Threonine</td>
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</tr>
<tr>
<td>7</td>
<td>Proline*</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Measured after Step 6 as prolinamide on the amino acid analyzer.

... served. Diphenylthiourea and phenylthiourea give peaks which elute earlier than the alanyl thiodyantoin, the most volatile derivative on all columns. One as yet unidentified peak elutes just after the leucyl derivative on columns containing DC-560. Another elutes close to the phenylalanyl PTH position on both DC-560 and XE-60 columns.

Columns containing DC-560 are recommended for Groups I and III, and a column containing XE-60 for Group II and for distinguishing the isoleucyl and leucyl derivatives. The silylation procedure is used for the analysis of the aspartyl, glutamyl, and cysteic acid compounds and for confirming the identity of other members of Group III. Silylation may also serve as a useful confirmatory procedure for Group II and I derivatives. Most phenylthiohydantoins readily form silyl derivatives in an apparently quantitative yield. The derivatives have better chromatographic properties and give greater responses in the flame ionization detector. However, it is not yet possible to recommend silylation of unknown samples exclusive of direct injection. Multiple product formation and variable reaction rates observed with some silylated derivatives and the appearance of silylated sample impurities make the direct injection of the phenylthiohydantoins advisable in many cases.

The two column system has proved to be a satisfactory method for identification of all the thiodyantoins except that of arginine; however, a single column system would obviously simplify and speed the technique. One approach, the use of blends of several silicone stationary phases, has given encouraging results. The DXO column which may be regarded as a prototype has given excellent chromatograms not only with standards but in practical applications as well. Its main limitations appear to be the poor resolution of isoleucyl and leucyl thiodyantoins and the silylated derivatives of aspartyl and asparaginyl PTHs. This deficiency in resolution should be overcome with new blends of stationary phases, or stacking in one column of packings containing different stationary phases.5,10

When considering the quantitative analysis of phenylthiohydantoins, it must be remembered that there are two separate parts of the problem, the Edman procedure and the gas chromatographic analysis. It is well known that certain amino acid phenylthiohydantoins (e.g. seryl, threonyl, asparaginyl, glutaminyl, histidyl, tryptophanyl, S-carboxymethylcysteinyl) are not obtained in quantitative yield in the Edman procedure. In addition, even stable derivatives are obtained in decreasing yields with each successive step of the degradation. Both the instability and decreasing yields are readily seen in the gas chromatographic technique (Table II). In this analysis, it should be noted that although certain derivatives undergo thermal decomposition and others give reduced responses due to adsorption to the column packing, remarkably reproducible results are obtained under a given set of conditions. Consequently, adsorption and instability are not serious limitations in the assay.

Phenylthiohydantoin standards, which have been observed to give consistently low responses on all columns tested, include members of Groups I and II. Employing the DXO column, the responses of the derivatives, relative to alanyl phenylthiohydantoin, were: threonyl, 0.90 (sum of both peaks); seryl, 0.50 (major peak); histidyl, 0.45; glutaminyl, 0.40; S-carboxymethylcysteinyl, 0.40; asparaginyl, 0.35; and lysyl, 0.15. The exact causes of these low responses are currently under investigation. With the S-carboxymethylcysteinyl and lysyl derivatives, the peaks represent degradation products. Some degradation is also evident with the other derivatives as well. The anhydro compounds have been seen with the seryl and threonyl derivatives, and asparaginyl and glutaminyl PTHs give minor early peaks. Repeated crystallization of the derivatives did not alter the results.

Attempts to analyze arginyl PTH have been unsuccessful, presumably because of the high polarity of the guanidinium group and the inability to form a stable silyl derivative (16). Fortunately, analysis of this derivative is relatively simple, as it is always found in the aqueous layer after the conversion step of the Edman procedure and may be quantitatively measured in solution by a micro-Sakaguchi test or qualitatively as a spot on paper or on a thin layer plate. The free amino acid may also be measured after hydrolysis of the derivative. Whatever procedure is selected, it is useful to remember that arginine is often the COOH-terminal amino acid when a tryptic peptide is sequenced.

When compared to the currently employed paper and thin layer chromatographic procedures, the gas chromatographic method offers superior resolving power, ease of quantitation, speed, and sensitivity. The limit of detection of Group II derivatives and some members of Group III was 0.1 μg, but as little as 0.02 μg of the others could readily be identified. This high sensitivity proved to be particularly valuable in studies on the structure of porcine thyrocalcitonin which was available in only extremely limited quantity (11). Finally, since the gas chromatographic procedure determines the thiodyantoin at each step of the degradation, it appears particularly well suited to the analyses of samples obtained from the automated protein sequenator of Edman.

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