Infrared Spectra of 3-Phenyl-2-thiohydantoins of Amino Acids and Their Application to Identification of *N*-Terminal Groups in Peptides

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Infrared spectra of the phenylthiohydantoins of 22 amino acids are recorded. A modified method has been used for preparing the phenylthiohydantoins of asparagine and glutamine. Experiments with five peptides demonstrate the usefulness of infrared spectra for positive identification of *N*-terminal amino acid residues.

THE thiohydantoins and phenylthiohydantoins of amino acids recently have assumed importance in amino acid sequence studies on peptides and proteins. Schlack and Kumpf (*14*) identify C-terminal amino acids in proteins as the 2-thiohydantoins. The determination of amino acid sequence in peptides and proteins by the method of Edman (*3*) involves characterization of the residues through the 3-phenyl-2-thiohydantoins. Methods have been developed for their chromatographic separation and identification, on specially treated papers (*10, 15*). The molecular extinction coefficients for the thiohydantoins in the region of 270 mμ are of considerable value in their quantitative determination, but the spectra of these compounds in the ultraviolet region are of little use for their identification (*5*).

This communication presents the infrared spectra of the 3-phenyl-2-thiohydantoins of 22 amino acids. A characteristic spectrum was obtained for each compound tested. The amino acids from which these compounds arose, therefore, could be identified positively. The N-terminal residues in five synthetic peptides were identified correctly by use of the above spectra.

EXPERIMENTAL METHODS AND RESULTS

Materials. PREPARATION OF 3-PHENYL-2-THIOHYDANTOINS OF AMINO ACIDS. The 3-phenyl-2-thiohydantoins of glycine, alanine, leucine, isoleucine, valine, methionine, proline, hydroxyproline, tryptophan, tyrosine, phenylalanine, 3,4-dihydroxyphenylalanine (not previously described), aspartic acid, glutamic acid, lysine, arginine, and histidine were prepared according to the method of Edman (*2*). Each compound was crystalline, and had a melting point in satisfactory agreement with that reported by Edman (*2*). It was not possible, however, to remove the yellow color associated with the derivative of tryptophan by repeated recrystallization from glacial acetic acid and water. The use of Edman's method for preparing the phenylthiohydantoin of asparagine yielded only the derivative of aspartic acid.

The identity of the compound was proved by melting point (*1, 2*), mixed melting point, and comparison of infrared absorption spectra. This indicated that the amide group of the phenylthiocarbamyl asparagine had been hydrolyzed during the conversion to the phenylthiohydantoin. By using milder conditions for this reaction, the phenylthiohydantoin of asparagine and also of glutamine was obtained easily. To assure preservation of the amide group, the procedure was modified as follows:

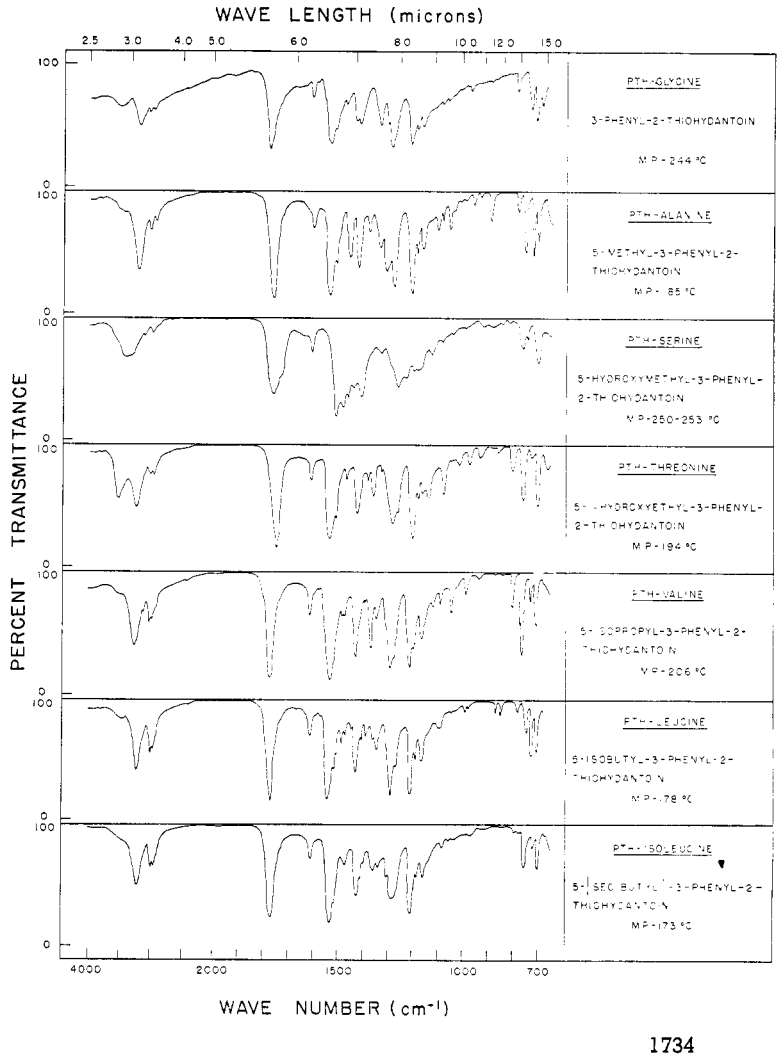


Figure 1. Infrared spectra of phenylthiohydantoins

Crystalline state

The reaction mixture containing the crude N-phenylthiocarbamyl derivative of asparagine was extracted repeatedly with benzene. It was then adjusted to pH 1 with 2*N* hydrochloric acid and allowed to stand at room temperature for 2 days. The crystalline material which had settled out was collected by filtration, after chilling, washed with water, and recrystallized from ethyl alcohol and water. The phenylthiohydantoin of asparagine so obtained had a melting point of 239° C. (found: carbon, 52.87; hydrogen, 4.448; nitrogen, 16.79; calculated: carbon, 53.01; hydrogen, 4.450; nitrogen, 16.87), and displayed the infrared absorption bands characteristic of the —CO—NH_2 grouping (two bands in the 3200- to 3400-cm.$^{-1}$ region and one other near 1660 cm.$^{-1}$). The phenylthiohydantoin of glutamine had a melting point of 218° C. (found: carbon, 54.88; hydrogen, 4.980; nitrogen, 15.85; calculated: carbon, 54.75; hydrogen, 4.978; nitrogen, 15.97).

Since the completion of this work the authors' attention has been drawn to a recent review (*6*) in which a general method using milder conditions for ring closure is advocated for the preparation of the phenylthiohydantoins of amino acids. The melting points of the derivatives of asparagine and glutamine were reported as 234° and 193° C., respectively. No elementary analyses of the compounds were given, and it is not clear whether the melting point tabulated for the phenylthiohydantoin of asparagine was obtained on a preparation by the method suggested, or was that found by Edman (*2*).

Phenylthiohydantoins of the hydroxyamino acids were prepared according to the method of Ingram (*8*). The phenylthiohydantoin of threonine (2-amino-3-hydroxybutyric acid) obtained had the reported physical constants. Difficulty was encountered in preparing the pure phenylthiohydantoin of serine (2-amino-3-hydroxy propionic acid). Different samples possessed widely different melting points. Some insoluble amorphous material separated during attempts to recrystallize the derivative from ethyl alcohol–water mixtures (*5*).

The phenylthiohydantoin of hydroxylysine, 5-[3-hydroxy-4-(β-phenylthioureido)butyl]-3-phenyl-2-thiohydantoin—not previously described—was prepared from a racemic sample of hydroxylysine isolated from gelatin (*11*). The derivative melted at 119° C. (found: carbon, 57.93%; hydrogen, 5.35%; calculated: carbon, 57.60%; hydrogen, 5.38%).

Instrumental. Measurement of Infrared Spectra. The infrared spectra were recorded with a Perkin-Elmer, Model 21 infrared spectrophotometer equipped with a rock salt prism. The compounds were mounted in potassium bromide (*13*, *17*). Two methods of sample preparation were applied: The pure crystalline compound (1 mg.) was thoroughly ground in a mortar with 500 mg. of powdered potassium bromide. The compound was first dissolved in a minimum amount of ethyl alcohol (about 0.5 ml.) and then mixed with potassium bromide. The mixture was dried in a vacuum desiccator over phosphorus pentoxide for 2 to 3 hours.

Both samples were pressed into windows for infrared recording.

The infrared spectra of the phenylthiohydantoins of the 22 amino acids are given in Figures 1 to 3.

Edman Degradation of Simple Peptides for Identification of N-Terminal Residues. Experiments were done to determine whether infrared spectra could be used to identify the N-terminal residues of some peptides through the phenylthiohydantoins. The following peptides were used: DL-alanylglycylglycine, L-leucyl-glycine, DL-phenylalanylglycine, DL-histidylhistidine, and γ-glutamylcysteinylglycine (glutathione). Reaction with phenyl isothiocyanate was carried out in 50% pyridine at pH 8.6. Ring closure and cleavage of the N-terminal residue as the phenylthiohydantoin was effected by anhydrous nitromethane–hydrogen chloride (*3*); the extract containing the phenylthiohydantoin derivative of the N-terminal amino acid was evaporated to dryness and the material recrystallized for identification. The procedure was applied to 10- to 20-mg. samples of the peptides, alanylglycylglycine, leucylglycine, and phenylalanylglycine. With all three the N-terminal residues were correctly identified, by comparison with the infrared spectra of phenylthiohydantoins of known amino acids. With 2.7 mg.

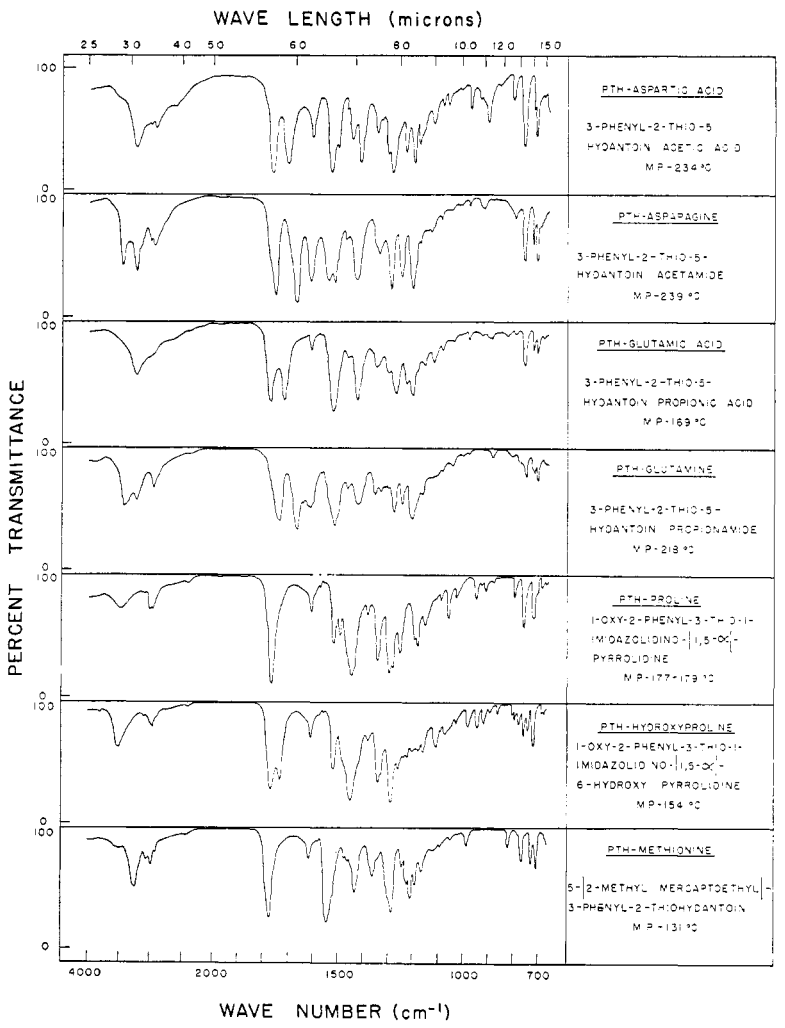


Figure 2. Infrared spectra phenylthiohydantoins

Crystalline state

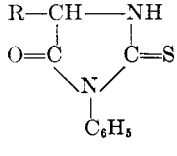
of histidylhistidine, the phenylthiohydantoin derivative could not be crystallized to yield a preparation devoid of yellow coloring matter. When 10-mg. amounts of histidylhistidine were processed, crystalline phenylthiohydantoin of histidine identical with a standard preparation in melting point and spectral characteristics was obtained.

Reaction of 10 mg. of glutathione with phenylisothiocyanate was 93% complete, as indicated by alkali consumption. With this peptide, however, the insoluble product obtained by treating the reaction mixture with nitromethane–hydrogen chloride was not the free phenylthiohydantoin of glutamic acid. Although ring closure had occurred the reagent did not hydrolyze the γ-glutamyl peptide bond. The product was therefore refluxed for 4 hours in 4*N* hydrochloric acid, and then extracted with ether. The residue from the ether extract after recrystallization possessed the same spectral characteristics as those of glutamic acid phenylthiohydantoin.

DISCUSSION

For many of the amino acid derivatives, the spectrum obtained by the second sampling technique was the same as obtained by the first method, where the sample was maintained in the crystalline state. For identification through the infrared spectra, it, therefore, is advisable to treat the crystalline derivatives, to be identified, by the first sampling technique. If, however, the derivative is obtainable only in solution, then the spectra of the known derivatives for reference should be obtained using the same sampling method as used for the unknown. Thus, when the second sampling technique was used, the phenylthiohydantoins of aspartic acid, glutamic acid, tryptophan, tyrosine, and lysine showed marked changes in their infrared absorption properties (Figure 4). The absorption bands were generally broader, slightly shifted in wave number, and weaker. In the case of aspartic and glutamic acids the two carbonyl bands (near 1775 and near 1700 cm.$^{-1}$), which are well resolved for the crystalline state, added up to form one broad band with a maximum near 1750 cm.$^{-1}$ Changes in infrared absorption properties, similar to the ones mentioned, are known to be caused by a change in the physical state of the substance (*7*, *9*, *12*). It appeared that the mentioned phenylthiohydantoins did not crystallize during the drying operation and remained in the liquid state. The same "liquid" spectra can be obtained after heating the potassium bromide window prepared by the first sampling technique to the melting point of the corresponding compound.

All spectra exhibited characteristic bands, which made it possible to recognize the phenylthiohydantoin ring (I) in the compounds.



R = H or side chains of the corresponding α-amino acids

These bands appeared in the following regions of the infrared spectrum:

1770 to 1740 cm.$^{-1}$ assigned to ring C=O vibration.
1600 cm.$^{-1}$ assigned to phenyl group vibration.
1425 to 1400 cm.$^{-1}$ assigned to C=S vibration.
3300 to 3150 cm.$^{-1}$, 1530 to 1500 cm.$^{-1}$ assigned to NH vibration (the N—H bands are not shown by the proline and hydroxyproline phenylthiohydantoins).

In the arginine derivative, the phenyl band at 1600 cm.$^{-1}$ is overlapped by the strong C=NH absorption. The N—H stretching vibration in the 3300 to 3150 cm.$^{-1}$ region is often modified by hydroxyl stretching vibrations of the derivatives of the hydroxy amino acids.

The identification of the phenylthiohydantoin derivative of an amino acid by infrared spectroscopy requires 1 mg. of the derivative, thus imposing a lower limit on the amount of peptide that may be degraded. In general, samples containing at least 1 mg. of a single N-terminal amino acid are required, and for convenience in handling, peptide samples of 10 to 20 mg. are desirable. For analysis of microquantities, the application of paper chromatography, on specially treated papers, is necessary. The latter method can be used to detect as little as 2 to 3 γ of phenylthiohydantoins of amino acids (*12*).

The identification of N-terminal amino acids in peptides or

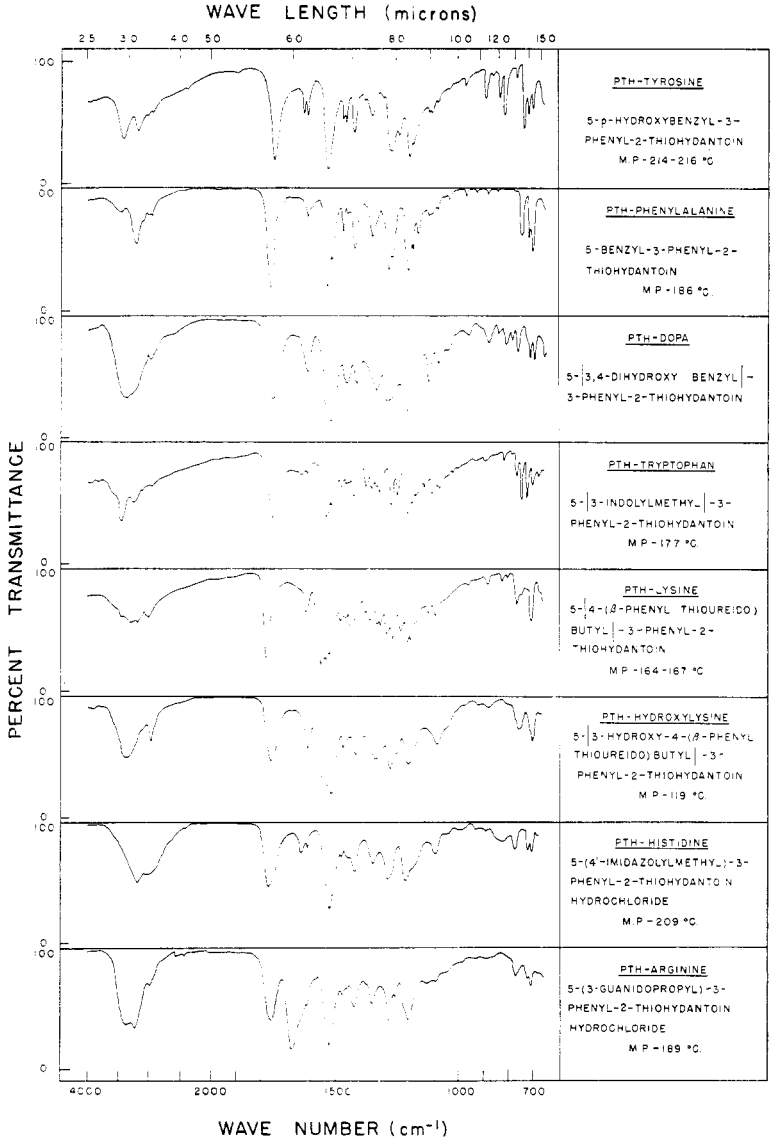


Figure 3. Infrared spectra of phenylthiohydantoins
Crystalline state

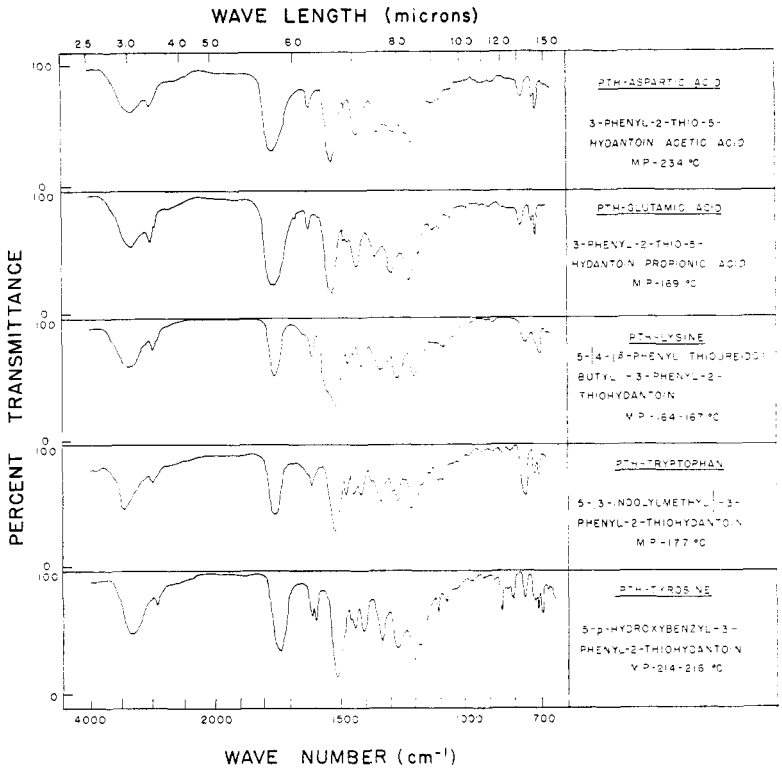


Figure 4. Infrared spectra of phenylthiohydantoins

Liquid state

proteins by the infrared spectra of their phenylthiohydantoin derivatives is also limited to samples containing a single peptide species that does not possess branched chains. Satisfactory methods for separation and purification of the phenylthiohydantoins of amino acids (*16*) and for peptides increase the usefulness of the method by materially extending the cases for which it is applicable. Where sufficient quantities of pure materials are available, the infrared method, as used herein, provides a simple and powerful means for the unambiguous identification of 3-phenyl-2-thiohydantoins of amino acids.

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High Precision Spectrophotometric Microanalysis with Application to Vanadium-Aluminum Alloys

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Optically matched cuvettes are necessary for existing high precision spectrophotometric methods. The maintenance of optical matching is not practical for routine analysis at high precision. Procedures are presented utilizing a modification of the Beer's law equation which permit the use of unmatched cuvettes for high precision spectrophotometric analysis. The errors brought about by the use of unmatched cuvettes are calculated and they indicate the optimum conditions, relative to mismatching, for the use of Hiskey's method. These procedures were applied to the analysis for vanadium in two vanadium-aluminum alloys. The accuracy obtained on synthetic samples averaged within 0.1%. The reproducibility on the actual samples averaged within 0.2%.

THE most economical analyses are those which are shortest and simplest in their execution. From this standpoint spectrophotometric analysis is very attractive. It is one of the leading methods for trace analysis but, because of its lack of reproducibility, has been little used for the determination of major components.

Ringbom (*10*), Hiskey (*5–7, 13*), and Bastian (*1, 2*) have shown that very good reproducibility may be obtained in spectrophotometric analysis if a solution of high absorbance is used for the blank. Hiskey (*5*) has shown that the error in comparing two solutions of similar absorbance may be as low as 0.04% based on concentration. This increased precision results from the use of the spectrophotometer at scale values allowing maximum

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