Proton Transfer Mass Spectrometry of Underivatized Peptides†

Robert J. Beuhler, Everett Flanigan, Lewis J. Greene, and Lewis Friedman*

ABSTRACT: Proton transfer spectra of the following underivatized peptides have been studied in a tandem mass spectrometer system: Ser-Phe-Pro, Met-Val-His, Leu-Trp-Met, Met-Met-Leu, Tyr-Ile-His-Pro-Phe, Gly-Phe-Ser-Pro-Phe, Phe-Asp-Ala-Ser-Val, Asp-Ser-Leu-Gly-Arg, <Gln-Lys-Trp-Ala-Pro, Trp-Met-Asp-Phe-NH_2, and Gly-Trp-Met-Asp-Phe-NH_2. With NH_4^+ as the ionizing reagent and rapid heating techniques used to evaporate samples dispersed on Teflon [Beuhler, R. J., Flanigan, E., Greene, L. J., and Friedman, L. (1974), *J. Amer. Chem. Soc. 96,* 3990 (1974)] fragmentation processes were observed primarily at peptide bonds. Peptides were run as unknowns at levels of ~2 nmol and all major peaks in the respective spectra were correlated with amino acid sequences.

The major obstacle to the general application of mass spectrometric techniques to sequencing of amino acid residues in peptides is the volatility problem. The relatively low volatility and thermal instability of peptides have led to the development of chemical derivatization techniques, *i.e.*, acetylation, permethylation, reduction, and arginine modification, to enhance peptide volatility by reducing electrostatic and hydrogen bonding interactions (Das and Lederer, 1971; Shemyakin *et al.*, 1971; Biemann, 1972; Van Lear and McLafferty, 1969; Fales *et al.*, 1972; Gray *et al.*, 1970; Nau *et al.*, 1973; Kiryushkin *et al.*, 1971; Morris *et al.*, 1974). However, general, simple, quantitative derivatization techniques suitable for peptides at the 1–10 nmol level have not yet been developed (*cf.* Fales, 1972).

Studies on underivatized peptides which show considerable promise have been made using field desorption mass spectrometry, a technique which integrates the evaporation and ionization process in the mass spectrometer ion source (Winkler and Beckey, 1972). Recently studies exploiting the gentle ionization feature of chemical ionization have demonstrated that it is possible to evaporate many underivatized dipeptides and obtain spectra useful for identification and sequencing (Bowen and Field, 1973). An imaginative variation of the chemical ionization technique that provides for direct introduction of liquids containing dissolved underivatized peptides into a chemical ionization source has been developed by McLafferty and coworkers (Baldwin and McLafferty, 1973a). This technique has made possible the observation of protonated parent molecule ions of a variety of peptides including Ala-Ala-Ala-His. Underivatized samples of hexalanine were run by direct insertion into a chemical ionization source (Baldwin and McLafferty, 1973b).

Our concern is primarily with the volatility problem. We shall present spectra of peptides that may have undergone significant decomposition in the course of evaporation prior to ionization. In those cases in which a fragmentation spectrum is observed without detection of a protonated parent molecule ion, volatility enhancement is not demonstrated. But if a spectrum of significant structural value is produced, the volatility problem is at least in part circumvented if not solved. This conclusion was supported in our recent study of arginine containing peptides (Beuhler *et al.*, 1974). The results presented in this paper we hope will stimulate efforts to adapt volatility enhancement techniques and rapid heating methods to more conventional single source gentle ionization mass spectrometer systems.

The purpose of this report is to demonstrate an extension of the technique to peptides containing a wider variety of amino acids and to indicate more generally the capabilities and limitations of rapid heating and single ion impact proton transfer ionization as a means of obtaining sequence information on underivatized peptides at the 1–10 nmol level. The tri-, tetra-, and pentapeptides documented here contain methionine, histidine, aspartic acid, glutamic acid, serine, phenylalanine, tryptophan, tyrosine, and lysine. They were chosen because these amino acids present somewhat more difficult volatility and thermal instability problems than the simpler aliphatic amino acids. In addition, these experiments provide confirmation and an extension of our previous observations on the cleavage, rearrangement, and elimination reactions connected with the rapid heating and single ion impact proton transfer ionization of peptides (Beuhler *et al.*, 1971,1972,1974).

Experimental Section

Materials—Peptides. Leu-Trp-Met and Phe-Asp-Ala-Ser-Val were purchased from Research Plus Laboratories and Trp-Met-Asp-Phe-NH_2 from Bachem Fine Chemicals. Met-Val-His, Met-Met-Leu, Gly-Trp-Met-Asp-Phe-NH_2, and Tyr-Ile-His-Pro-Phe were gifts from Schwarz/Mann. Ser-Phe-Pro, Gly-Phe-Ser-Pro-Phe, and <Gln-Lys-Trp-Ala-Pro were synthesized by the solid phase method (Stewart and Young, 1969) and purified by gradient elution chromatography on Dowex 50-X2 (Schroeder, 1967). <Gln-Lys-Trp-Ala-Pro was acetylated using 50% aqueous acetic anhydride (Hodges *et al.*, 1972) four times to ensure complete acetylation. The extent of the acetylation reaction was monitored by high voltage electrophoresis at pH 6.5. Asp-Ser-Leu-Gly-Arg was prepared from human pancreatic secretory trypsin inhibitor (Pubols *et al.*, 1974) and the amino acid sequence was independently determined by sub-

† From the Departments of Biology and Chemistry, Brookhaven National Laboratory, Upton, New York 11973. *Received June 14, 1974.* Research was carried out under the auspices of the U. S. Atomic Energy Commission.

tractive Edman degradation and enzymatic hydrolysis (Greene *et al.*, 1974).

The peptides had the expected amino acid composition after acid hydrolysis in 6 N HCl, *i.e.*, integral molar ratios of the constituent amino acids. No free amino acids were detected in excess of 1% (mol/mol) when the peptides were subjected to amino acid analysis without prior hydrolysis. The peptides behaved as single components when subjected to high voltage electrophoresis, pH 3.5 and 6.5. Ninhydrin and the chlorine–tolidine reagent (Pataki, 1968) were used for detection of the peptide.

Apparatus and Procedures. Details of the proton transfer, rapid heating technique used to obtain the spectra presented in this report have been published recently (Beuhler *et al.*, 1974). Gentle ionization of neutral gaseous species was achieved with NH_4^+ gaseous ions generated in the first stage of our tandem mass spectrometer. Peptide samples (1–5 nmol) were dispersed from dilute H_2O solutions onto a Teflon-covered probe which was heated rapidly at rates up to 12°/sec after insertion into a Teflon-lined collision chamber. Mass analysis of product ions was obtained with a computer controlled quadrupole system capable of scanning preselected mass ranges with dwell times as short at 2 msec per mass point. Sample evaporation times were typically limited to a few seconds.[1]

Results

We shall present mass spectra giving relative abundances of various product ions plotted as a function of *m/e*. The ions presented in the spectra appeared in a narrow time and temperature band during the course of heating the sample.

Tripeptides. The mass spectra of four tripeptides, Ser-Phe-Pro (Figure 1a), Met-Val-His (Figures 1b), Leu-Trp-Met (Figure 1c), and Met-Met-Leu (Figure 1d) conform to a general pattern. There is at the high end of the spectrum a protonated parent molecule ion. Intense peaks corresponding to the carboxyl-terminal residue as a protonated amino acid and an intense amino-terminal dipeptide fragment peak are observed in each spectrum. The sequence of the tripeptide is established by a somewhat weaker set of ions corresponding to a protonated carboxyl-terminal dipeptide and its dehydrated derivative, corresponding in mass to the protonated diketopiperazine. The only other ions significantly above noise shown in Figure 1a–d can be accounted for by decomposition processes involving loss of small stable molecules, *e.g.*, H_2O (*m/e* 18); NH_3 (*m/e* 17); CO (*m/e* 28); H_2O + CO or HCOOH (*m/e* 46). For example, the *m/e* 207 peak in the Ser-Phe-Pro spectrum (Figure 1a) is accounted for by the loss of CO from the protonated (Ser,Phe) fragment peak (*m/e* 235). Similarly, in Met-Val-His (Figure 1b) the high molecular weight ions at *m/e* 351 and 350 are accounted for by the loss of H_2O and NH_3 or $2H_2O$ molecules from the protonated parent ion. The loss of methylmercaptan (*m/e* 48) from the (Met, Val) fragment ion (*m/e* 231) accounts for the ion at *m/e* 183. The loss of the elements of formic acid (*m/e* 46) from the carboxyl-terminal histidine (*m/e* 156) accounted for the ion at *m/e* 110. In the Leu-Trp-Met spectrum (Figure 1c) there is a relatively intense peak at *m/e* 130 corresponding to methylindole derived from the trytophan residue and a relatively small peak at *m/e* 141 which we have been unsuccessful in rationalizing.

The ability to identify most of the significant ions in the spectrum is important because it permits the introduction of arguments based on the failure to observe carboxyl-terminal ion fragments derived from the other amino acid residues in the molecule. In addition, for tripeptides failure to observe dipeptides containing the N- and C-terminal amino acid residues constitutes important sequence information.

The peptide Met-Met-Leu (Figure 1d) illustrates a problem arising from isobaric masses of combinations of amino acids. In the figure *m/e* 263 is identified as the protonated (Met,Met) dipeptide fragment. The protonated C-terminal dipeptide H-Met-Leu-OH also has a *m/e* 263. In the tripeptide spectra presented in Figure 1a–c, cleavage of the N-terminal peptide bond produced relatively weak ion intensities. The abundance of the ion at *m/e* 263 suggests that it is primarily Met-Met. Similarly, an argument can be made for the identification of *m/e* 132 as carboxyl-terminal Leu rather than anhydro Met. These identifications were confirmed by a study of the isotopic peaks of sulfur in the ions at *m/e* 132, 134, 263, and 265, respectively. The abundance of ions at *m/e* 134 is less than 1% of *m/e* 132 and much too low to account for the natural abundance of ^{34}S (~4%). The isotopic peak at *m/e* 265 corresponds to ~9% of the peak at *m/e* 263, indicating two S atoms in the ion or a Met-Met structure. The peak at *m/e* 264 is accounted for by the natural abundance of ^{13}C, ^{15}N, ^{17}O, and deuterium. A small correction for double-labeled ions is required to obtain a quantitative result at *m/e* 265 that establishes the two sulfur atoms in the ion.

Tetrapeptide and Pentapeptides. The problem of identification of species in the spectra of larger peptides (Figures 1e–l) is in fact no more difficult than that encountered with the tripeptides. Since these spectra are more complex, we have included a set of neutral molecule fragmentation schemes which show hypothetical mechanisms for generation of neutral fragments that on protonation can account for the ions in the respective spectra. The use of a neutral

[1] It has been noted in review that the effect of the Teflon on rates of evaporation of peptides deserves special comment. Assumption of a uniform deposit of a few nanomoles of peptide on a surface area of a few tenths of a square centimeter leads to the conclusion that the peptide is deposited in approximately 100 molecular layers on the Teflon surface. If indeed there were a uniform deposit of 100 layers of peptide molecules it might be difficult to account for the observed effects. Two points, however, must be considered: one is the relation between the actual surface of the probe to the macroscopic geometric surface and the other is the question of the uniformity of the deposit of peptides. We know very little about the epitaxial effect of Teflon on the habit of crystals produced on the probe. It is, however, fairly safe to conclude that in view of the relatively weak interaction between the peptide and Teflon there will be a strong tendency for peptide molecules to aggregate rather than to disperse in a smooth uniform layer. Furthermore, the actual surface of the Teflon is indeed porous as revealed by low resolution scanning electron microscope examination. There is adequate space (probably 100 times the macrosurface area) for a large number of microcrystals with molecules located at base edges bonded relatively weakly to the crystal and weakly to the Teflon. These molecules evaporate first, always leaving more "edge" molecules behind. If the surface interacted more strongly with the peptide then the "edge" molecules would be less vulnerable to evaporation. We note these remarks are speculative but indeed somewhat less speculative than the assumption that the Teflon has a microscopically smooth surface and that the peptide molecules spread out smoothly on this surface. They provide a qualitative rationalization for the initiative required to carry out the experiments. It may well be that, as has been pointed out in review, we are dealing with evaporation from peptide surfaces which when deposited on Teflon have higher volatility because of differences in the crystal structure produced by epitaxial effects. The problem of the volatility of samples deposited in less than 100 molecular layers must be reserved for further physical study. The authors are grateful to Dr. Myron Ledbetter of the BNL Biology Department for providing them with electron microscope pictures of the Teflon that has been used in this work.

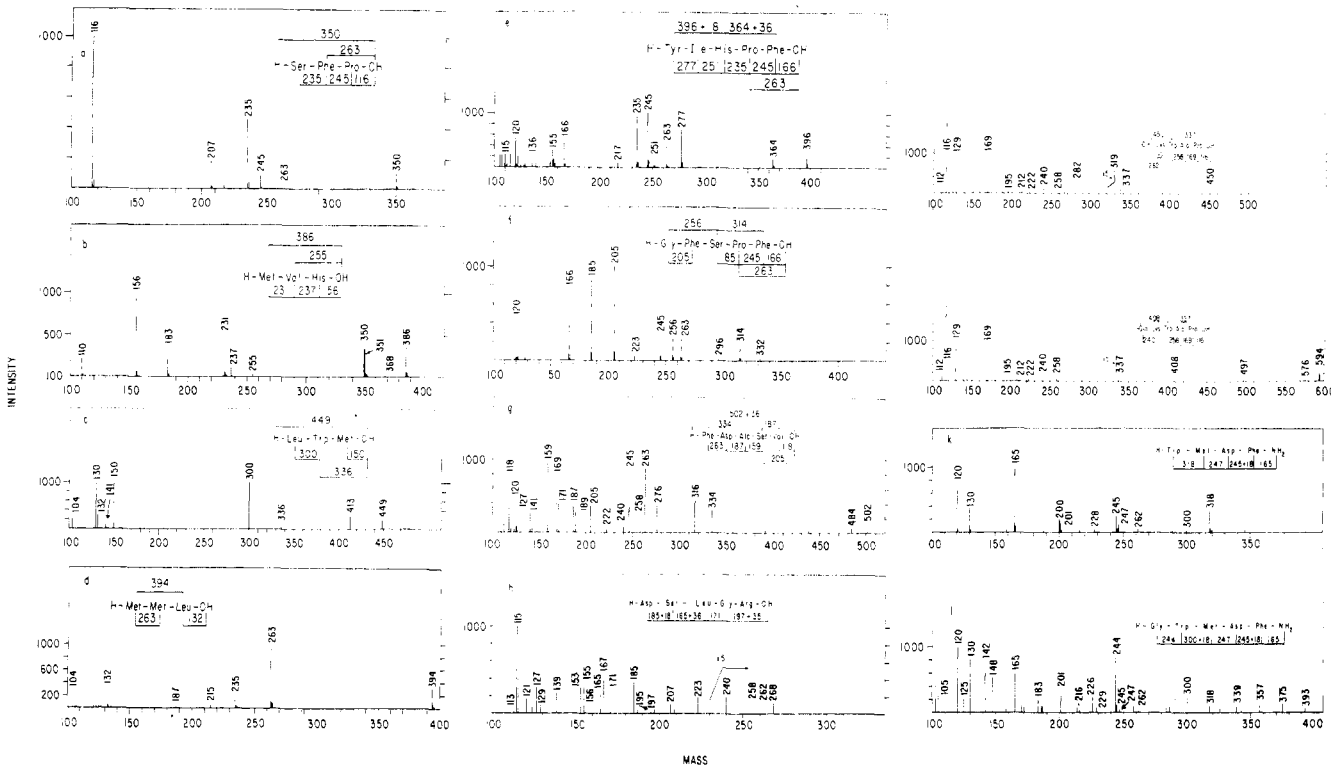

FIGURE 1: (a) Proton transfer spectrum of H-Ser-Phe-Pro-OH, primary ionizing beam ~1 eV NH_4^+. Sequence peaks are identified in the inset. *m/e* 207 not identified in the inset is Ser-Phe, *m/e* 235, minus CO. Values of the ordinate are in arbitrary units with the most abundant peak normalized to a value of 1000. (b) Proton transfer spectrum of H-Met-Val-His-OH. Masses not identified in the inset are 368, 351, and 350 formed by loss of water, water and ammonia, and two water molecules, respectively, from the protonated parent molecule ion. *m/e* 183 is identified as the product of loss of CH_3SH from Met-Val. *m/e* 110 produced by loss of 46 mass units (HCOOH) from *m/e* 156. (c) Proton transfer spectrum of H-Leu-Trp-Met-OH. *m/e* 413 formed by loss of $2H_2O$ from parent, *m/e* 132 is the anhydro Met ion (loss of H_2O from *m/e* 150), *m/e* 130 is a methylindole residue from tryptophan, and *m/e* 104 formed by loss of 46 mass units from C-terminal Met. Sequence ions are identified in the inset. (d) Proton transfer spectrum of H-Met-Met-Leu-OH. The isobaric ion H-Met-Leu-OH, *m/e* 236, is not indicated in the inset. The *m/e* 235 ion is formed by loss of CO from N-terminal Met-Met. *m/e* 215 is identified as the fragment at 235 minus water or the loss of CH_3SH from *m/e* 263. Loss of CO from this latter fragment gives *m/e* 187. *m/e* 104 is identified as loss of CO from a methionine residue. (e) Proton transfer spectrum of H-Tyr-Ile-His-Pro-Phe-OH. See Figure 2 for the identification of possible neutral fragment precursors of ions not identified in the inset. (f) Proton transfer spectrum of H-Gly-Phe-Ser-Pro-Phe-OH. See Figure 3 for identification of fragments not indicated in inset. (g) Proton transfer spectrum of H-Phe-Asp-Ala-Ser-Val-OH with NH_4^+. See Figure 4 for identification of fragments. (h) Proton transfer spectrum of H-Asp-Ser-Leu-Gly-Arg-OH. See Figure 7 for fragmentation scheme. (i) Proton transfer spectrum of acetylated <Gln-Lys-Trp-Ala-Pro-OH. (j) Proton transfer spectrum of underivatized <Gln-Lys-Trp-Ala-Pro-OH. See Figure 5 for fragmentation scheme. (k) Proton transfer spectrum of H-Trp-Met-Asp-Phe-NH_2. (l) Proton transfer spectrum of H-Gly-Trp-Met-Asp-Phe-NH_2. See Figure 6 for fragmentation scheme.

fragmentation scheme in contrast to ion fragmentation emphasizes the possibility of peptide decomposition prior to ionization and provides for a concise presentation of complementary dissociation processes which satisfy mass balance. Complementary processes could be shown from protonated species by indicating separate reaction channels but this would add to the complexity of the figures. Ions in the mass spectra are generally identified by adding one mass unit to the neutral fragments in decomposition schemes.

The following conditions must be satisfied if fragment ion mass spectra is to be used for sequencing small underivatized peptides: (1) sufficient volatility must be achieved to generate a spectrum that can be uniquely correlated with an amino acid sequence; (2) there must be sufficient overlap information in the fragment ions; (3) isobaric fragment ions cannot be used without independent supplementary information. The peptide mass spectra presented below illustrate cases of (a) an "easy" pentapeptide, Tyr-Ile-His-Pro-Phe; (b) a relatively volatile pentapeptide in which an overlap element was not observed, Gly-Phe-Ser-Pro-Phe; (c) examples of pentapeptides containing isobaric depeptide fragments, Phe-Asp-Ala-Ser-Val and <Gly-Lys-Trp-Ala-Pro; (d) an example of the change in spectrum observed by the addition of glycine to the N-terminal position of Trp-Met-Asp-Phe-NH_2; and finally (e) an example of a difficultly volatile peptide Asp-Ser-Leu-Gly-Arg.

Tyr-Ile-His-Pro-Phe (Figures 1e and 2). The sequence was determined on the basis of the abundant ions corresponding to dipeptides and the carboxyl-terminal phenylalanine residue as indicated in Figure 1e. With the exception of ions *m/e* 155 and 136, the decomposition scheme in Figure 2 provides a basis for the identification of the bulk of the mass spectra.[2]

Gly-Phe-Ser-Pro-Phe (Figures 1f and 3). Figure 1f shows that Ser-Pro-Phe is the carboxyl-terminal sequence and a Gly-Phe or Phe-Gly amino terminal element. The Gly,Phe,Ser tripeptide element ties the di- and tripeptide fragments together but does not establish the Phe-Ser or

[2] The *m/e* 136 ion could be formed by loss of H_2O and the benzyl radical from protonated Pro-Phe fragment and *m/e* 155 may be derived from His,Pro by the loss of the imidazole ring and the CH attaching to the peptide chain. A variety of water elimination reactions are observed in the fragmentation schemes some of which involve loss of carbonyl oxygen (*e.g.*, formation of *m/e* 217 from the His-Pro dipeptide and *m/e* 367 from the His-Pro-Phe tripeptide). These processes are not common in electron impact spectra of ketones or similar molecules. They may be examples of processes unique to the single ion impact proton transfer processes or the water elimination may take place in the rapid heating evaporation of the adsorbed neutral molecules.

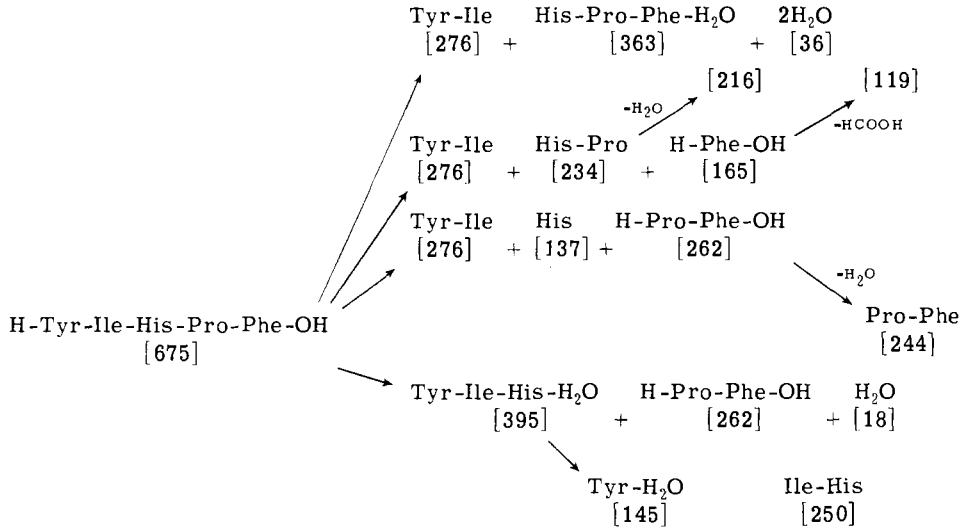

FIGURE 2: Fragmentation scheme for neutral H-Tyr-Ile-His-Pro-Phe-OH showing possible products of unimolecular decomposition of samples heated in spectrometer collision chamber. Masses of respective fragments are indicated in brackets. These masses are for neutral unprotonated species, one mass unit below values of ionic masses shown in Figure 1e. Each horizontal row gives a set of complementary neutral species.

Gly-Ser linkage required for a complete sequence determination. The only basis available in the data for making a choice between a Gly-Phe-Ser or a Phe-Gly-Ser element is in the argument that *m/e* 332, 314, and 296 can be derived from Phe-Ser-Pro as well as Ser-Pro-Phe. With no evidence for a Gly-Ser-Pro fragment, the Gly-Phe-Ser-Pro-Phe structure was considered a more likely choice in that it included both Phe-Ser-Pro and Ser-Pro-Phe tripeptides.

Phe-Asp-Ala-Ser-Val (Figures 1g and 4). This peptide illustrates problems of mass ambiguities in spectra of peptides with the dipeptide fragments (Asp,Ala) and (Ser,Val), *m/e* 187, unresolved in low-resolution studies. There is an additional problem at *m/e* 189 with carboxyl-terminal Ala-Val and the Phe-Asp fragment, formed by loss of elements of formic acid and CO, having the same mass. These are both cases in which high-resolution mass spectrometry could have valuable application.

The mass spectrum establishes carboxyl terminal valine as an anchor for the sequence by the intense peak at *m/e* 118 and failure to observe the alternative C-terminal serine, alanine, aspartic acid, or phenylalanine. The relatively intense peaks at *m/e* 263 and 245 suggest that (Phe,Asp) is probably the N-terminal dipeptide. While *m/e* 189 could be taken as evidence for carboxyl terminal (Ala,Val,fragment) and the 171 peak interpreted as evidence for the ion corresponding to the protonated Ala-Val fragment, *m/e* 205 corresponds to C-terminal Ser-Val and *m/e* 187 its dehydrated derivative. The choice of serine as the penultimate residue is based on observation of the (Ala,Ser) peak at *m/e* 159 and the attachment of Ala to the (Phe,Asp) dipeptide indicated by *m/e* 334 and *m/e* 316. The latter ions are identified as (Phe,Asp,Ala) and (Phe,Asp,Ala, $-H_2O$). Since Ala can have only two points of attachment in the peptide chains, the Ala-Ser-Val sequence element is deduced as being the correct assignment in spite of the mass ambiguities cited above. With no evidence for a Phe-Ala linkage at *me/* 219 and the (Asp,Ala) or (Ser,Val) *m/e* 187 peak, the sequence Phe-Asp-Ala-Ser-Val provides the highest level of internal consistency with the observed spectrum. An alternative sequence placing alanine adjacent to the C-terminal valence would have left the peak at *m/e* 205 unexplained and would have required the sequence element Asp-Ala-Val to account for *m/e* 187. This would lead to a Ser-Phe-Asp-Ala-Val sequence. Failure to observe the Ser-Phe dipeptide and its dehydrated derivative at *m/e* 235 and 217, respectively, and Ser-Phe-Asp peaks provides additional arguments against this alternative.

<Gln-Lys-Trp-Ala-Pro. The mass spectrum of the pentapeptide is given in Figure 1j. The decomposition scheme given in Figure 5 contains fragments from the N-terminal

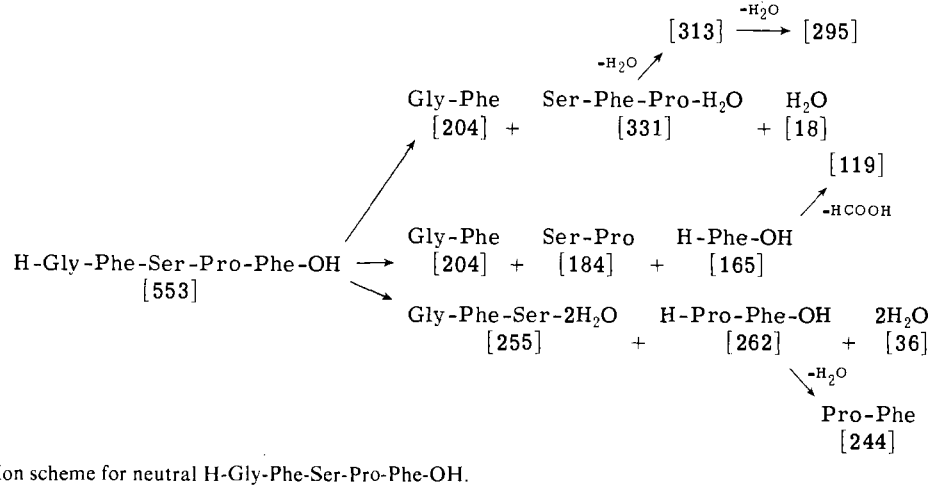

FIGURE 3: Fragmentation scheme for neutral H-Gly-Phe-Ser-Pro-Phe-OH.

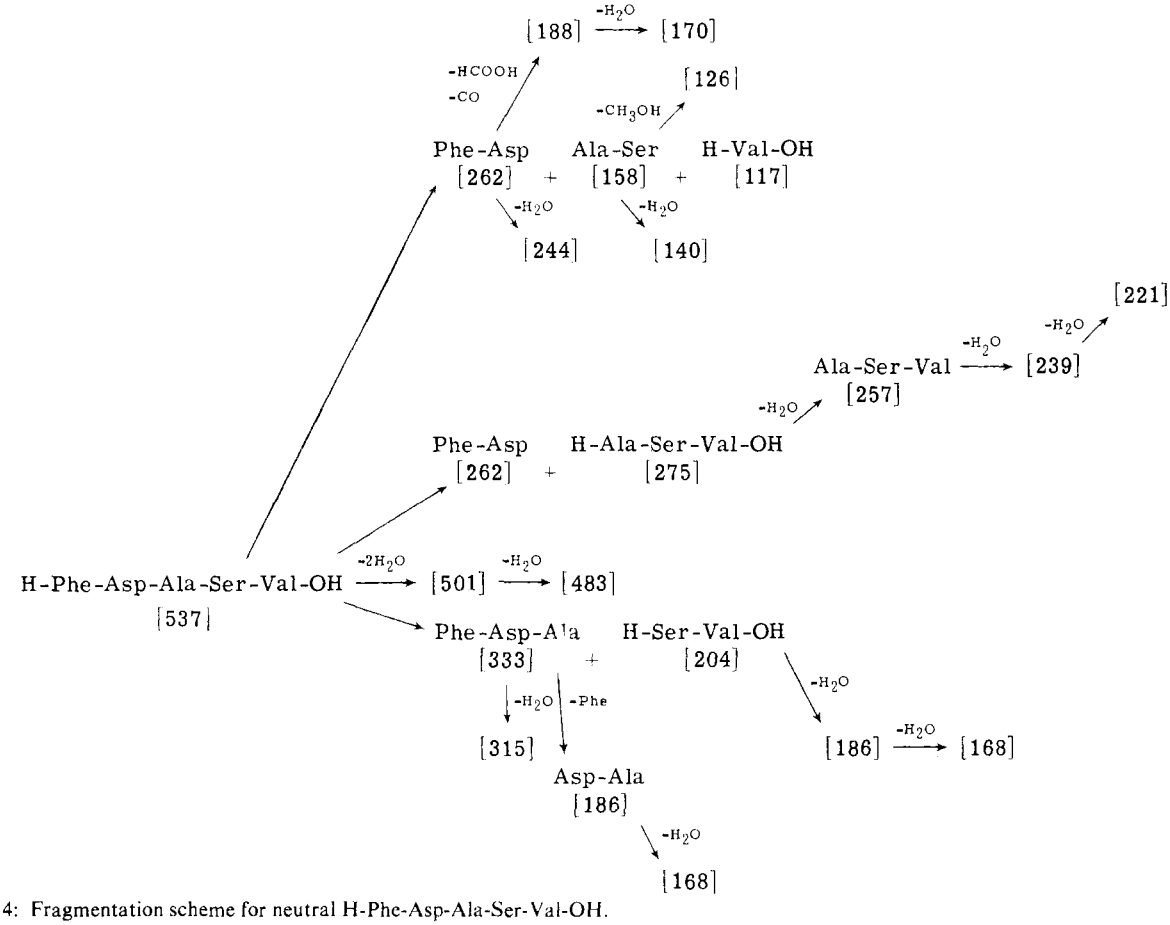

FIGURE 4: Fragmentation scheme for neutral H-Phe-Asp-Ala-Ser-Val-OH.

portion of the peptide *m/e* 129, 240, and 408 which have the same mass as ions observed in the spectrum. Furthermore the mass balance obtained by comparing the sums of complementary fragments for the three main decomposition channels shown in Figure 5 is high by one mass unit. This unusual behavior of fragments containing N-terminal <Gln suggests that either a hydrogen atom rearrangement takes place in the neutral decomposition process producing a proton deficient neutral fragment which upon ionization regains the mass that would be expected from simple peptide bond rupture or these fragments are formed exclusively by ionic decomposition processes of larger protonated species. If this behavior is observed generally it may provide a basis for the identification of N-terminal <Gln. The high mass region shows peaks at 595, 594, and 576, which are formed by the loss of ammonia and/or water or water molecules from the protonated parent molecule ion. The *m/e* 497 peak can be correlated with loss of a proline residue and water

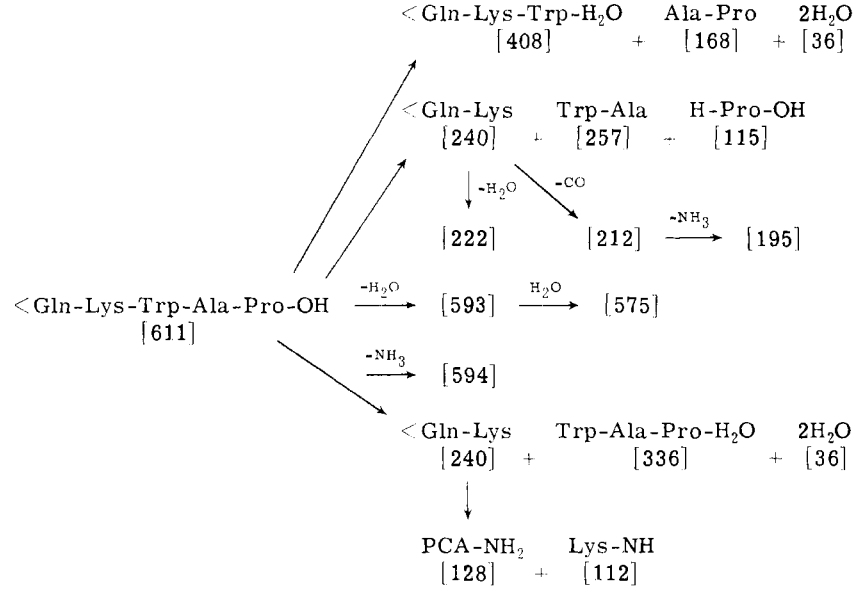

FIGURE 5: Fragmentation scheme for neutral <Gln-Lys-Trp-Ala-Pro-OH.

from the parent molecule ion. C-terminal proline is indicated by the *m/e* 116 peak. Alternative possibilities for C-terminal residues were not observed for alanine (*m/e* 90, not shown), lysine (*m/e* 147), glutamic acid (*m/e* 148), and tryptophan (*m/e* 205).

An unusual feature of this spectrum is that aside from the carboxyl-terminal proline peak at *m/e* 116 there is only one intense dipeptide ion peak, *m/e* 169, corresponding to (Ala,Pro). Were it not for the identification of proline as carboxyl-terminal, (Ala,Pro) would have been considered by virtue of its intensity a good candidate for the amino terminal dipeptide fragment. The intense *m/e* 129 peak is our best alternative for an N-terminal residue and it can be identified as either the protonated lysine residue (anhydro) or protonated <Gln amide derived from amino terminal glutamic acid or <Gln. This ambiguity was found to be unresolvable with the data available in the spectrum of the underivatized pentapeptide. The spectrum did show, however, evidence for a (Trp,Ala,Pro $- H_2O$) tripeptide fragment (*m/e* 337). In additon, the *m/e* 408 peak gave evidence for (Glu,Lys,Trp, $- H_2O$). The respective (Trp,Ala) and (Glu,Lys) linkages are further supported by the peaks at *m/e* 258 and 240 which can be shown to be precursors of *m/e* 222, 212, and 195 (*cf.* Figure 5, top). The underivatized peptide spectrum supports either H-Glu-Lys-Trp-Ala-Pro-OH or H-Lys-Glu-Trp-Ala-Pro-OH as possible sequences.

A successful attempt was made to resolve the ambiguity in sequence and the uncertainty in the state of hydration of the Glu residue (*i.e.*, the question of whether the peptide contains <Gln, Gln, or Glu) by determination of the mass spectrum of the acetylated derivative. The spectrum shown in Figure 1i is remarkably similar to that of the underivatized peptide with new peaks appearing at *m/e* 450, 319, and 282 and the disappearance of the 408 peak. The *m/e* 319 peak appears to be the result of the further dehydration of the (Trp,Ala,Pro) residue. The loss of *m/e* 408 and the formation of the *m/e* 450 peak shows the introduction of *one* acetyl group in the (Glu,Lys,Trp) tripeptide. There is no evidence for the presence of more than one acetyl group in the Glu-Lys dipeptide. The 240 peak remains in the spectrum of the acetylated material because of the Trp-Ala-H_2O peak. The new peak at *m/e* 282 shows that some of the 240 in the spectrum of underivatized peptide was indeed <Gln-Lys.

The introduction of only one acetyl group into the peptide on acetylation is consistent with a <Gln-Lys element in the sequence with the free ϵ-amino group of the lysine the sole site for acetylation. The *m/e* 129 peak is thus identified as <Gln-NH_2. Fragmentation of this type with cleavage of an N-terminal <Gln-NH_2 was also observed in the spectrum of thyrotropin releasing hormone (<Gln-His-Pro-NH_2) (Beuhler *et al.*, 1972).

Gly-Trp-Met-Asp-Phe-NH_2. The spectrum of the pentapeptide is shown in Figure 1l with the spectrum of Trp-Met-Asp-Phe-NH_2 in Figure 1k. The latter spectrum is considerably simpler than that of the pentapeptide containing an additional glycine residue. The differences in spectra reflect more extensive decomposition of the less volatile pentapeptide. Both spectra show evidence of C-terminal Phe-NH_2 at *m/e* 165. An intense *m/e* 120 peak is formed by loss of the elements of $HCONH_2$ (or CO, NH_3) from this carboxyl terminal residue. A *m/e* 130 peak is frequently observed in spectra of peptides containing tryptophan.

Both spectra contain ions corresponding to the dipeptide fragements (Trp,Met) (*m/e* 318, 300), Met-Asp (*m/e* 247), and (Asp,Phe $- H_2O$) (*m.e* 245). The overlap of these dipeptide fragments taken with identification carboxyl-terminal Phe-NH_2 and supported by the intensity of *m/e* 318 peak indicating probable N-terminal position for Trp-Met gives the sequence Trp-Met-Asp-Phe-NH_2 for the tetrapeptide. The relatively intense *m/e* 244 (Gly,Trp) peak, in the pentapeptide not seen in the tetrapeptide, extends the amino acid sequence to Gly-Trp-Met-Asp-Phe-NH_2.

The decomposition scheme given in Figure 6 can be used to identify ions observed in the mass range 120 and above in the pentapeptide spectrum. The rather intense peaks at *m/e* 125, 142, and 148 in the lower mass region of the pentapeptide spectrum not seen in the tetrapeptide spectrum are identified as decomposition products of Asp-Phe-NH_2 or Phe-NH_2. These decomposition processes are not observed at the lower temperatures required for the evaporation of the tetrapeptide or its fragment. The decrease in the relative intensity of Trp-Met (*m/e* 318) and corresponding increase in Trp-Met anhydro (*m/e* 300) is another example of the perturbation brought about by the necessity of higher temperatures for the evaporation of the pentapeptide. The sum of the intensities of *m/e* 318, 300 in the pentapeptide spectrum is still considerably lower than the *m/e* 244 peak which corresponds to the N-terminal fragment in this molecule. The most intense dipeptide fragment in the tetrapeptides is the Trp-Met (*m/e* 318) peak. Generally the spectra are well accounted for by the assumption of the unimolecular decomposition of parent molecules with initial bond rupture processes taking place at peptide bonds, followed by elimination of small neutral stable molecules. The only exceptions to be noted are the processes associated with *m/e* 142 and 125 in which toluene is assumed to be cleaved from carboxyl-terminal Asp-Phe-NH_2 along with H_2O, CO, and NH_3 molecules (*cf.* Figure 6). The relatively minor tripeptide peaks and their derivatives in the mass range 318–373 are shown to be completely consistent with and in fact support the sequence assignment.

Asp-Ser-Leu-Gly-Arg. The outstanding feature of the spectrum (Figure 1h) is the intense *m/e* 115 peak which, taken with the amino acid composition of this pentapeptide, indicates carboxyl-terminal arginine. Earlier work on proton transfer mass spectra of arginine and carboxyl-terminal arginine peptides shows the loss of elements of urea from the C-terminal arginine giving an intense peak corresponding to a protonated ornithine lactam (*m/e* 115) (Beuhler *et al.*, 1974). This conclusion is supported by the absence of ions corresponding to carboxyl-terminal aspartic acid (*m/e* 134), leucine (*m/e* 132), serine (*m/e* 106), and glycine (*m/e* 76, not shown in Figure 1h).

The most intense peaks corresponding to dipeptide fragments which we would speculate to be the N-terminal dipeptide are found at *m/e* 185 and 167 (Asp-Ser $- H_2O$, Asp-Ser, $- 2H_2O$). Evidence for a (Ser,Leu) dipeptide is found at *m/e* 165 corresponding to the loss of $2H_2O$ from the protonated (Ser,Leu) fragment. (Leu,Gly) attachment is indicated by the *m/e* 171 peak. The dipeptide peaks cited, taken with evidence for C-terminal arginine and the intensity argument for N-terminal (Asp,Ser), suggest the sequence Asp-Ser-Leu-Gly-Arg. The peaks at *m/e* 258, 240, and 223 are identified as the Ser-Leu-Gly fragment and its dehydration and deamination products (*cf.* Figure 7). This mass assignment supports the conclusions drawn from the dipeptide fragments.

Evidence for the (Asp,Ser,Leu) tripeptide is found at *m/e*

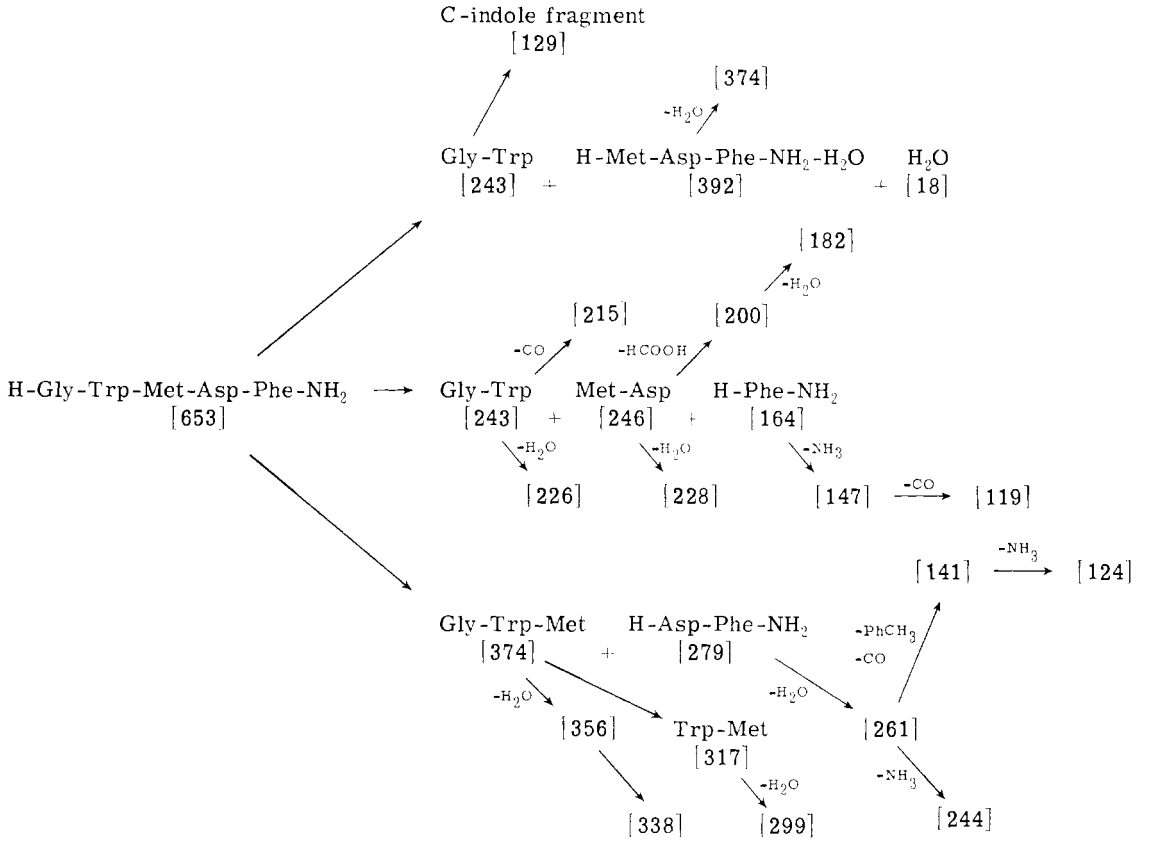

FIGURE 6: Fragmentation scheme for neutral H-Gly-Trp-Met-Asp-Phe-NH_2. The species produced from the similar fragmentation of H-Trp-Met-Asp-Phe-NH_2 have been included.

262 (Asp,Ser,Leu, $-3H_2O$) and *m/e* 207 [Asp,Ser,Leu $-$ (CO, H_2O, NH_3, HCOOH)]. The (Leu,Gly,Arg) tripeptide is indicated by *m/e* 268 [H-Leu-Gly-Arg-OH $-$ (urea + NH_3)]. These relatively small peaks which have undergone extensive decomposition are cited only as a supplementary support of the sequence derived from the more intense ion yield in the spectrum. It is, however, important to note the consistency of the minor peaks in the spectrum with the sequence assignment. The weakest point in the sequence assignment is the linkage of glycine to carboxyl-terminal arginine. The *m/e* 197 is the peak directly related to carboxyl-terminal Gly-Arg (*m/e* 232). There is a set of peaks in the dipeptide region of the spectrum at *m/e* 153, 155, and 156 which can be assigned structures corresponding to decomposition products of C-terminal (Gly,Arg) (*cf.* Figure 7). The alternative of an (Asp-Arg) linkage with the sequence H-Gly-Leu-Ser-Asp-Arg-OH is not supported by observation of C-terminal (Asp,Arg) (*m/e* 290) or fragments corresponding to loss of urea, water, NH_3, etc.

The lower mass peaks in the spectrum that are not identified in Figure 7 can be shown to be decomposition products of arginine or the (Asp,Ser) dipeptide fragment.

Discussion

The fundamental difficulty in the application of structural mass spectrometer techniques to sequencing peptides is identified as the volatility problem and is associated with the nondestructive evaporation of neutral peptide molecules which can be ionized to produce a mass spectrum that can be correlated with the structure of the parent neutral molecule. Success in this correlation is based largely on a close correlation of parent molecule ion structures with parent neutral molecule structures and the fact that the excited parent neutral molecule ions decompose unimolecularly as isolated species with limited and generally predictable molecular rearrangements processes. It is certainly not safe to assume *a priori* that decompositions taking place in the course of the evaporation of solid samples of neutral peptide molecules are unimolecular processes. Fragments generated by reaction of two or several peptides could lead to significant errors in the assignment of an amino acid sequence. A more obvious problem with peptide mass spectra is the generation of a sufficient concentration of neutral species in the gas phase to provide an observable mass spectrum from the limited samples of material frequently available in natural product studies.

A potential solution to these problems is found in the application of rapid heating of small samples of peptides dispersed on inert surfaces. The idea of dispersal of small samples on Teflon surfaces was initially pursued as a volatility enhancement technique which was designed to minimize interactions between molecules of peptides. Rapid heating of the sample was shown to favor evaporation of parent species taking place in competition with fragmentation processes on the solid surface. Both techniques, *i.e.*, dispersal on inert surfaces and rapid heating, minimize the interaction of molecules on the surface and enhance the probability of the evaporation of either parent neutral molecules or fragments which are produced by the unimolecular decomposition of peptide molecules on the surface. Such conditions were achieved and correlations of spectra of the products with structure of parent neutral molecules on the surface have been shown to have a quality of correlation very similar to that obtained for mass spectra of stable gaseous species. With the substitution of gentle nondestructive proton trans-

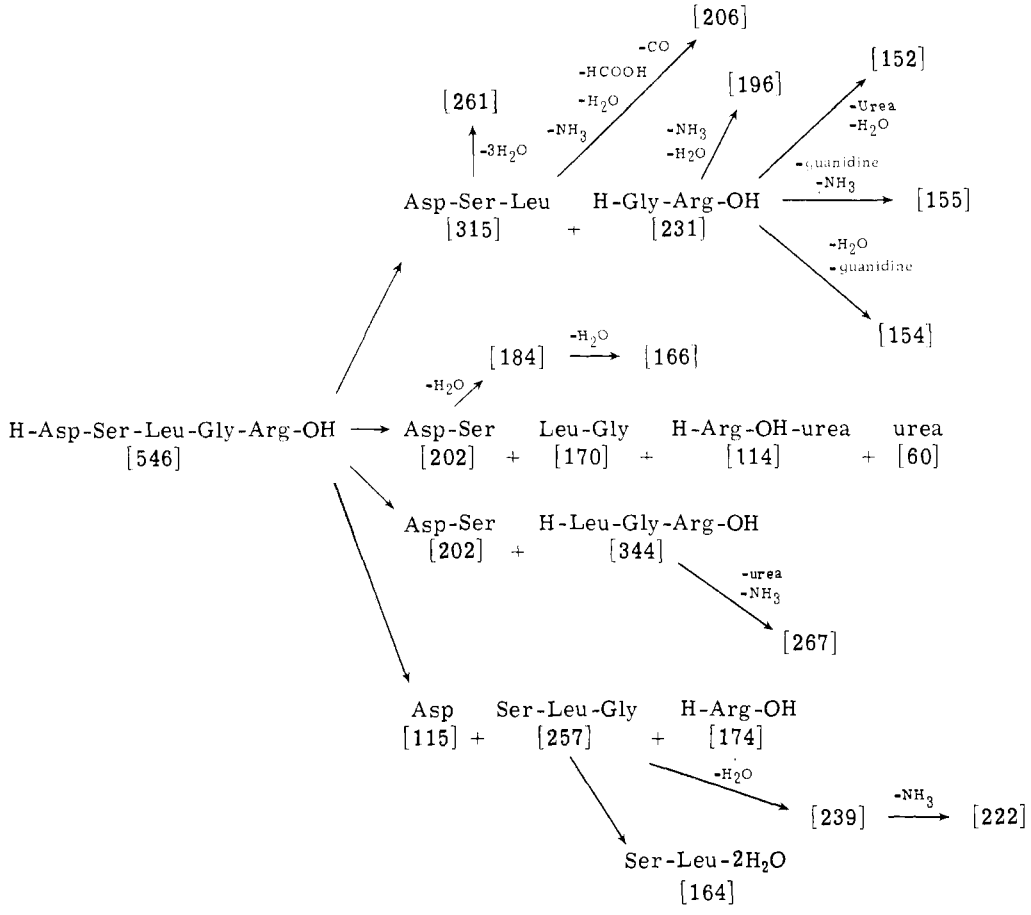

FIGURE 7: Fragmentation scheme for neutral H-Asp-Ser-Leu-Gly-Arg-OH.

fer ionization for the more conventional electron impact methods the question of fragmentation prior or subsequent to ionization becomes less important if one can demonstrate that the fragmentation process can be shown to be reproducible and well correlated with parent molecule structures. Furthermore we have no evidence of rearrangement of amino acid residues within the peptide chain taking place during evaporation.

A very important feature of the kinetics of unimolecular decomposition processes that applies to decomposition of excited molecules ions (electron impact mass spectra) and the decomposition of many neutral molecules is the role of product stability in determining reaction rate. When the activated intermediates in a unimolecular decomposition are structurally similar to the reaction products, competitive decomposition processes will favor the formation of stable products. Consequently, elimination of water, NH_3, CO, HCOOH, urea, guanidine, etc., is expected along with hydrogen atom rearrangements that produce stable neutral and ionized systems. The analysis of the spectra presented here is based on these concepts. These results, which account for essentially all of the observed ions, may be taken as support for validity of this description of the system. Similar considerations have been applied in our earlier study of arginine containing peptides with similar results. In the present work, however, the consideration of N-terminal dipeptide intensity was used as an additional basis for identifying these sequence elements. The peptides studied include a wide variety of amino acid residues which provide both difficulty in evaporation and occasionally mass ambiguities in the analysis of the data. We note that all of the peptides studies here were treated as unknowns with the only input to structure analysis of mass spectral data being the amino acid composition of acid hydrolysates. The mass spectral data served to identify the amides in Trp-Met-Asp-Phe-NH_2 and Gly-Trp-Met-Asp-Phe-NH_2. In the case of the <Gln-Lys-Trp-Ala-Pro and the Asp-containing peptides Phe-Asp-Ala-Ser-Val and Asp-Ser-Leu-Gly-Arg no evidence for Gln or Asn derivatives was observed. The N-terminal PCA residue in <Gln-Lys-Trp-Ala-Pro might be difficult to distinguish from N-terminal Glu and future work with model compounds may shed light on this point. Acetylation was required to distinguish between these two forms of glutamic acid. At present the method does not appear capable of distinguishing between Ile and Leu residues. The gentle ionization technique which serves to minimize fragmentation would probably not produce sufficient decomposition of the isomeric side chains.

The ability to run underivatized material provides a significant sensitivity advantage. The sample size required for these studies is sensitively dependent on the volatility of the molecules being investigated. With most tripeptides and volatile pentapeptides such as Tyr-Ile-His-Pro-Phe samples significantly smaller than the nominal nanomole sample size suffice for low-resolution mass spectra. For less volatile compounds, *e.g.*, Gly-Trp-Asp-Met-Phe-NH_2, identification of the sequence may require several runs thereby consuming up to 10 nmol. To the best of our knowledge the only other work reported on underivatized peptides has been either by field desorption (Winkler and Beckey, 1972) or the recent studies of Bowen and Field (1973), Baldwin and McLafferty (1973a,b), and Bogentoft *et al.* (1972).

Their results generally demonstrate the superior sensitivity of mass spectra obtained with underivatized materials

Acknowledgments

The authors wish to express their appreciation to Schwarz Mann, Orangeburg, N.Y., a Division of Becton, Dickinson and Co., for making available many of the peptides used in this work and D. C. Bartelt for samples of the pentapeptide Asp-Ser-Leu-Gly-Arg derived from human pancreatic secretory trypsin inhibitor. The authors are grateful to Professor F. W. McLafferty for valuable constructive comments on this manuscript.

References

Baldwin, M. A., and McLafferty, F. W. (1973a), *Org. Mass Spectrom. 7,* 1111.

Baldwin, M. A., and McLafferty, F. W. (1973b), *Org. Mass Spectrom. 7,* 1353.

Beuhler, R. J., Flanigan, E., Greene, L. J., and Friedman, L. (1972), *Biochem. Biophys. Res. Commun. 46,* 1082.

Beuhler, R. J., Flanigan, E., Greene, L. J., and Friedman, L. (1974), *J. Amer. Chem. Soc. 96,* 3990.

Beuhler, R. J., Greene, L. J., and Friedman, L. (1971), *J. Amer. Chem. Soc. 93,* 4307.

Biemann, K. (1972), *in* Biochemical Applications of Mass Spectrometry, Waller, G. R., Ed., New York, N.Y., Wiley-Interscience, p 405.

Bogentoft, C., Change, J.-K., Sievertsson, H., Currie, B., and Folkers, K. *(1972), Org. Mass Spectrom. 6,* 735.

Bowen, D. V., and Field, F. H. (1973), *Int. J. Peptide Protein Res. 5,* 435.

Das, B. C., and Lederer, E. (1971), *in* New Techniques in Amino Acids, Peptide and Protein Analysis, Niederwieser, A., and Pataki, E., Ed., Ann Arbor, Mich., Ann Arbor Science Publishers, p 175.

Fales, H. M. (1972), *Recent Progr. Horm. Res. 28,* 625.

Fales, H. M., Milne, G. W. A., Pisano, J. J., Brewer, H. B., Jr., Blum, M. S., MacConnell, J. G., Brand, J., and Law, N. (1972), *Recent Progr. Horm. Res. 28,* 591.

Gray, W. R., Wojcik, L. H., and Futrell, J. H. (1970), *Biochem. Biophys. Res. Commun. 41,* 1111.

Greene, L. J., Roark, D. E., and Bartelt, D. C. (1974), *in* Bayer-Symposium, 6th, Fritz, H., Tschesche, H., Greene, L. J., and Truscheit, E., Ed., Heidelberg, Germany, Springer-Verlag (in press).

Hodges, R., Kent, S. B. H., and Richardson, B. C. (1972), *Biochim. Biophys. Acta 257,* 54.

Kiryushkin, A. A., Fales, H. M., Axenrod, T., Gilbert, E. J., and Milne, G. W. A. (1971), *Org. Mass Spectrom. 5,* 19.

Morris, H. R., Batley, K. E., Harding, N. G. L., Bjur, R. A., Dann, J. G., and King, R. W. (1974), *Biochem. J. 137,* 409.

Nau, H., Kelley, J. A., and Biemann, K. (1973), *J. Amer. Chem. Soc. 95,* 7162.

Pataki, G. (1968), *in* Techniques of Thin-Layer Chromatography in Amino Acid and Peptide Chemistry, Ann Arbor, Mich., Ann Arbor Science Publishers, p 407.

Pubols, M. H., Bartelt, D. C., and Greene, L. J. (1974), *J. Biol. Chem. 249,* 2235.

Schroeder, W. A. (1967), *Methods Enzymol. 11,* 351.

Shemyakin, M. M., Ovchinnikov, Y. A., and Kiryushkin, A. A. (1971), *in* Mass Spectrometry Techniques and Applications, Milne, G. W. A., Ed., New York, N.Y., Wiley-Interscience, p 289.

Stewart, J. M., and Young, J. D. (1969), Solid Phase Peptide Synthesis, San Francisco, Calif., W. H. Freeman.

Van Lear, G. E., and McLafferty, F. W. (1969), *Annu. Rev. Biochem. 38,* 298.

Winkler, H. U., and Beckey, H. D. (1972), *Biochem. Biophys. Res. Commun. 46,* 391.