Proton Transfer Mass Spectrometry of Peptides. A Rapid Heating Technique for Underivatized Peptides Containing Arginine[1]

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Abstract: Volatility enhancement and rapid heating techniques were used to obtain mass spectra of underivatized Arg-Pro, Arg-Pro-Pro, Arg-Pro-Pro-Gly, Arg-Pro-Pro-Gly-Phe, Ser-Pro-Phe-Arg, Pro-Phe-Arg, and Phe-Arg peptides. The advantage of rapid heating in establishing conditions favoring evaporation over competitive decomposition reactions is illustrated. Fragmentation processes in the evaporation of peptides and decomposition of protonated parent molecule ions are limited mainly to rupture of peptide linkages and elimination of small stable neutral molecules. Hydrogen atom rearrangements are frequently observed. The mass spectrum of bradykinin Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg was studied and spectra–structure correlations were sufficient to derive a unique amino acid sequence.

Mass spectrometry provides a rapid sensitive approach to problems of identification and structure determination of a variety of complex molecules, if they can be converted to gaseous ions. This volatility condition has been a limiting factor in attempts to use mass spectrometry to determine amino acid sequences in peptides. Chemical derivatization[2-7] techniques have been used to enhance the volatility of peptides by reducing hydrogen bonding interactions. Gaseous peptide ions have been directly evaporated in mass spectrometer ion sources by field desorption[8] techniques.

The volatility problem exists primarily because of the instability of peptide molecules at temperatures required for their evaporation. Energy required to break bonds attaching the peptides to a solid surface is distributed among internal degrees of freedom and absorbed in decomposition reactions. From the standpoint of structure determination mass spectra of fragments still provide much useful information. Nevertheless it is desirable to minimize fragmentation if mass spectra are to be correlated with neutral molecule structures. Field desorbed gaseous ions appear with very little fragmentation demonstrating a technique which deposits energy almost exclusively in bonds holding the molecule to the surface. The success of field desorption in minimizing the equilibration of energy among internal degrees of freedom of complex molecules points up the kinetic nature of evaporation processes which can take place at low pressures in a mass spectrometer ion source.

Consideration of the kinetics of the evaporation of peptides leads to two approaches to volatility enhancement. An obvious direction to take is to attempt to reduce the energy of bonding of molecules to surfaces by dispersal onto surfaces which are relatively inert. Volatility enhancement by dispersal of peptides on Teflon has been studied[9] using Thyrotropin Releasing Hormone (TRH) (PCA-His-Pro-NH_2) as a model compound. Underivatized polyalanine peptides up to and including pentaalanine[10,10a] have been evaporated from Teflon probes in Teflon collision chambers and have been shown to give relatively abundant yields of protonated parent molecule ions when bombarded with solvated protons in a tandem mass spectrometer system. This desorption technique is sensitive to trace impurities of nonvolatile compounds which coat the inert surface and bind the peptides strongly. It is limited because of the tendency of molecules to aggregate so that evaporation can take place in part from inert surfaces and in part from microcrystals produced in the dispersion of the sample on the surface.

A second approach to volatility enhancement, based on consideration of the kinetics of competitive evaporation and decomposition processes, is "rapid heating." The rate at which energy is deposited in a solid sample can be shown to enhance the yield of neutral gaseous molecules over fragments produced by surface decomposition processes. This is illustrated in the mass spectrum of TRH (PCA-His-Pro-NH_2) obtained by evaporating a solid sample from a copper probe in a Teflon collision chamber.[9] The protonated parent molecule ion, *m/e* 363, is a measure of the concentration of neutral gaseous TRH produced in the evaporation process. The fragment ion *m/e* 235 can be formed by decomposition of the protonated parent molecule ion by loss of pyrrolidinone carboxyl amide from the PCA-His-Pro-NH_2 or can be generated from species produced by a surface decomposition reaction. The extent

(1) Research performed under the auspices of the U. S. Atomic Energy Commission.

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

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

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

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

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

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

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

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

(10) Unpublished results of this Laboratory.

(10a) NOTE ADDED IN PROOF. Evidence for the evaporation of hexaalanine and other underivatized peptides in a chemical ionization source has come to our attention after submission of this paper. McLafferty and coworkers have reported signal to noise ratios of the protonated hexaalanine molecule ion greater than 100. *Cf.* Baldwin and McLafferty, *Org. Mass Spectrom.*, 7, 1353 (1973).

of fragmentation of the gaseous protonated parent ion is established by a study of evaporation of TRH from a Teflon probe. In these experiments during most of the heating cycle *m/e* 235 is found to be less than 20% of the intensity of *m/e* 363. The spectrum obtained by evaporation of TRH from a copper surface is quite different. Figure 1 shows plots of the log of relative ion intensity *vs.* reciprocal absolute temperature for the *m/e* 363 and *m/e* 235 ions from TRH. Initially and through the lower temperature stage of the evaporation process the *m/e* 235 ion is more abundant than the protonated parent ion. Slopes of the Arrhenius plots in Figure 1 show a lower activation energy for the overall process of formation and evaporation of *m/e* 235. If the sample is held at a temperature of ~160° ($1/T \cong 2.3$) the ratio of fragment 235 to protonated parent ion *m/e* 363 is approximately 3:1. If the sample is rapidly heated to ~215°, the protonated parent yield is more than twice that of the fragment ion. At this temperature and with these relative ion yields the ratio of protonated parent to fragment must be corrected for gaseous ion decomposition processes. If a correction is made for 20% parent to fragment decomposition then the higher temperature ratio of protonated parent to fragment is set at roughly 3:1. The abundance of the neutral parent and fragment species cannot be estimated precisely without knowledge of proton transfer ionization cross sections. However, it is reasonable to assume that these cross sections are roughly constant over the temperature range 150–225° or if there is a temperature sensitivity of cross section that to a first approximation the effects will cancel in the comparison of the two species. With these assumptions we can argue that the relative abundance of neutral TRH molecules in the gas phase is enhanced by nearly an order of magnitude by heating the copper probe rapidly to the higher temperature, 215°.

The concept of rapid heating as a technique to enhance the volatility of complex fragile species is based on a kinetic analysis of the competitive decomposition and evaporation processes. If the rate of evaporation of a neutral fragment is determined by an activation energy for decomposition, which is lower than the parent molecule evaporation activation energy, then Arrhenius plots of relative ion abundances *vs.* $1/T$ must intersect at some value of $1/T$. The fragment species produced with lower activation energy may be strongly favored at lower temperatures but if the parent molecular species can survive temperatures above the intersection of the respective Arrhenius plots then it will be more abundant in the gas phase at the higher temperatures.

A precise estimate of the extent of decomposition *vs.* evaporation at any temperature cannot be made without knowledge of ionization cross sections which are not available at present. In spite of this lack of basic information, the ability to produce relatively simple mass spectra with a minimum of surface decomposition justifies the investigation of rapid heating techniques.

In this report we describe the use of a system capable of heating samples at rates of roughly 10°/sec and a data acquisition system with total scan times of 2–3 sec and minimum dwell times at each mass position of 2 msec. Mass spectra will be presented of underivatized arginine and arginine-containing peptides including bradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. Correlations of spectra and structures will be discussed.

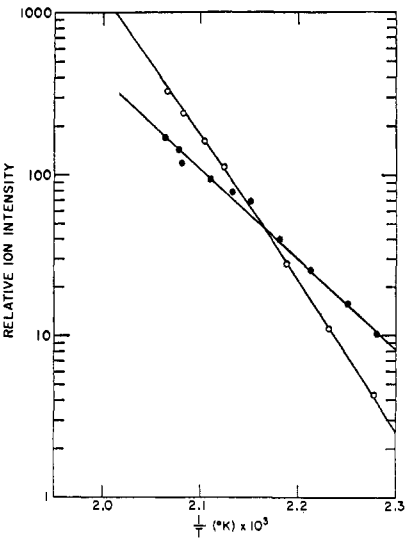


Figure 1. Relative intensity of *m/e* 363 and *m/e* 235 ions from TRH plotted as a function of $1/T$. Spectrum was obtained by evaporation of TRH from a copper probe surface in a Teflon collision chamber: (○) protonated parent molecule of PCA-His-Pro-NH_2, *m/e* 363; (●) *m/e* 235 ion formed by loss of pyrrolidinone carboxyl amide from PCA-His-Pro-NH_2.

Experimental Section

Apparatus and Procedures. A tandem mass spectrometer was used to obtain single ion impact mass spectra. The first stage mass spectrometer, a 12 in. radius 60° sector electromagnet, selects a particular primary ion reagent beam from the mixture of ions emitted from a high-pressure ion source. This mass analyzed primary ion beam enters a heated Teflon-lined collision chamber containing the vaporized peptide sample which has been evaporated from a Teflon-covered sample inlet probe inserted into the side of the collision chamber. The mechanism and energy of the ionization process can be controlled by the choice of primary ion beam species.[11] The proton transfer reaction of NH_4^+ with arginine containing peptides was investigated in this study. After the proton transfer reaction, the quasi-parent ion and fragment ions are extracted from the collision chamber. The mass spectrum of these secondary ions is then obtained in the second stage consisting of a quadrupole mass spectrometer (5.5 in. long, 0.25 in. diameter rods) and ion detector (Bendix Channeltron Model 4028).

A rapid heating system for the sample probe has been developed to minimize the effect of competitive decomposition processes during the evaporation process. The probe consists of a 0.075 in. diameter copper rod $1\frac{1}{8}$ in. long with a 0.002-in. Teflon foil wrapped over the tip. A clean piece of Teflon tied to the end of the probe with a 0.012-in. copper wire was used for each run. Peptide samples (1–5 nmol) were dispersed onto the Teflon from a dilute H_2O solution and dried under helium. This probe could be quickly inserted into the vacuum system *via* a vacuum lock. A molybdenum wire coil serves as the probe heater and holder, positioning the tip of the probe just inside the heated (150°) Teflon-lined collision chamber. With an input of up to 3 W into the heater, the rate of sample heating could be varied up to 12°/sec.

With the rapid sample heating, it was necessary to provide for rapid scanning of the spectrum and data acquisition. A PDP8/E computer (Digital Equipment Corporation) with 16 K of memory operates the power supply of the quadrupole mass spectrometer (Extranulcear Laboratories) and also collects the ion detector output as a function of quadrupole mass setting and scan number. A preselected scan sequence typed into the computer terminal controlled the output of a 14-bit DAC from 0.5 to 10 V which established the mass setting of the quadrupole from *m/e* 40 to 800. The output pulses of the Bendix multiplier are amplified and passed

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

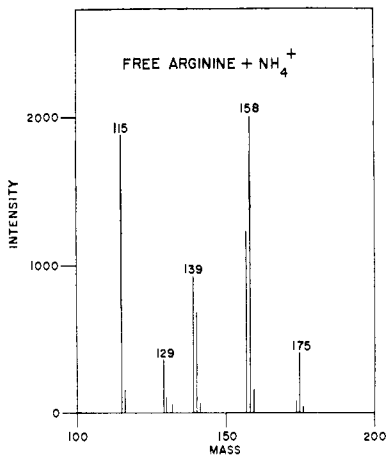


Figure 2. Proton transfer mass spectrum of arginine ionized with NH_4^+. The Teflon-lined collision chamber was held at 150°. The Teflon covered sample probe containing 1–2 nmol of arginine was heated at an estimated rate of 5°/sec. The values of the ordinate are in arbitrary intensity units.

through a 12-bit scaler into the computer memory where the number of counts in each mass bin for each scan is stored. Data are taken continuously for a selected number of scans using two 1024 word buffers; while one buffer is collecting data, the other empties onto Dec tape in 500 msec. The total information storage from a single run is limited to the 186,880 word storage on a Dec tape.

The computer control of the quadrupole mass analyzer was programmed to scan spectra in preselected mass ranges with a preselected number of points within each mass range. The number of points of observation in each mass range was defined by the step size. The minimum step size was 0.14 mass unit and the minimum dwell time per point was 0.002 sec. Total scan times on the order of 2.5–3.5 sec are used with a sample heating rate of 12°/sec. With these conditions, approximately 10 passes through the entire spectrum could be obtained before complete evaporation of the sample.

At the end of the scanning operation, the data are plotted on a Tektronix 4010-1 scope. Two forms of data output were found to be the most useful. The first, a display of ion intensity, is the number of counts observed in a selected mass range summed over a number of scans. The second is the intensity of a selected mass peak *vs.* scan number. The latter can be used for observing volatility differences in mixtures of peptides or other temperature (time)-dependent phenomena, and also for measurements of relative cross sections.

A primary beam of NH_4^+ having an intensity of 1×10^{-8} A (6×10^{10} ions/sec) generated yields of secondary ions ranging from 10^5 to 10^6 counts/nmol with a noise level of 5–10 cps. Therefore in the survey scans using 1–2 nmol of sample the total counts in a moderate peak was 1000 counts above noise when 100 preselected mass bins are scanned.

Reagents. A. Sources. Arg-Pro (1872) and Phe-Arg (4373) were purchased from Fox Chemical Co. Arg-Pro-Pro (023046) and Arg (1593) were products of Cyclo Chemical Co. and E. Merck (Darmstadt), respectively. Arg-Pro-Pro-Gly, Arg-Pro-Pro-Gly-Phe, Ser-Pro-Phe-Arg, and Pro-Phe-Arg were gifts from Dr. J. M. Stewart, University of Colorado Medical Center, Denver. Bradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, was purchased from JEM Research Products or Schwarz/Mann.

B. Characterization of Peptides. 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% (mole/mole) when the peptides were subjected to amino acid analysis without prior hydrolysis. The peptides were examined by high-voltage electrophoresis at pH 3.5 and pH 6.5 and detected by the chlorine–tolidine reagent.[12]

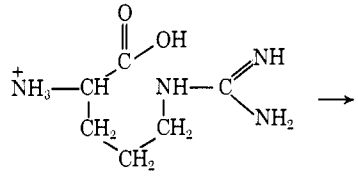
C. Partial Acid Hydrolysis of Bradykinin. Bradykinin (0.4 μmol) was sealed in an evacuated heavy walled tube with 1 ml of 2, 4, or 6 *N* HCl and held at 110° for 5–20 min. The sample containing bradykinin and fragments of bradykinin was chromatographed on the amino acid analyzer under conditions similar to those described by Camargo, *et al.*[13] The identification of hydrolysis products was based on the chromatographic behavior of synthetic peptides which were also used to determine color yields with the ninhydrin reagent.

(12) G. Pataki in "Techniques of Thin-Layer Chromatography in Amino Acid and Peptide Chemistry," Ann Arbor Science Publishers Inc., Ann Arbor, Mich., 1968, p 107.

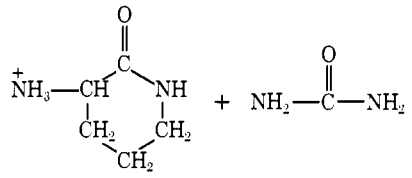
Discussion of Results

Mass Spectra of Arginine. Evidence for the evaporation of neutral gaseous arginine molecules based on the observation of a M + H peak at *m/e* 175 was not obtained until heating rates were increased to permit complete evaporation of a sample of a few nanomoles of arginine in less than 30 sec. With slower heating rates decomposition fragments corresponding to loss of NH_3 and H_2O at *m/e* 158 and 157, respectively, were the heaviest species detected. Ions at *m/e* 115 and 60 were also found in high yield. One might conclude that the very high proton affinity of arginine is responsible for gas phase decomposition of the M + H^+ ion formed by proton transfer from NH_4^+. This may be in part the case, but the spectra presented in Figure 2 obtained by collisions of NH_4^+ with arginine show that if the sample is heated rapidly, M + H^+ ions at *m/e* 175 are easily detected. The fragment ions formed by loss of NH_3, H_2O at *m/e* 158 and 157 are still present in large yield. Fragments at *m/e* 140 and 139 result from the loss of NH_3 and H_2O or $2H_2O$, respectively. The ion at *m/e* 129 is produced by the elimination of the elements of HCOOH from the protonated parent. This elimination is a favored process in the proton transfer spectra of amino acids.[14a]

The *m/e* 115 ion is found in abundant yield in the arginine spectrum and will be shown later to be an important component of spectra obtained from carboxyl terminal arginine containing peptides. The correlation of *m/e* 115 ions with C-terminal arginine peptides and



M + H^+ = 175



m/e 115

the free amino acid structures suggests the above concerted mechanism for the process, with the ener-

(13) A. C. M. Camargo, R. Shapanka, and L. J. Greene, *Biochemistry*, **12**, 1838 (1973).

(14) (a) G. W. H. Milne, T. Axenrod, and H. M. Fales, *J. Amer. Chem. Soc.*, **92**, 5170 (1970). (b) It has been suggested in review that the credibility of our mechanisms and correlations of mass spectra with structure might be improved by additional studies with isotopically labeled peptides and high-resolution mass spectrometry. With our present technique high-resolution mass spectrometry was not considered feasible. Isotopic tracer work with deuterium was not considered potentially fruitful because of the ubiquity of hydrogen rearrangements. Interesting and useful isotopic studies might be carried out with ^{15}N, ^{18}O, or ^{13}C labeled amino acid residues in these peptides. Unfortunately these materials were not available to us.

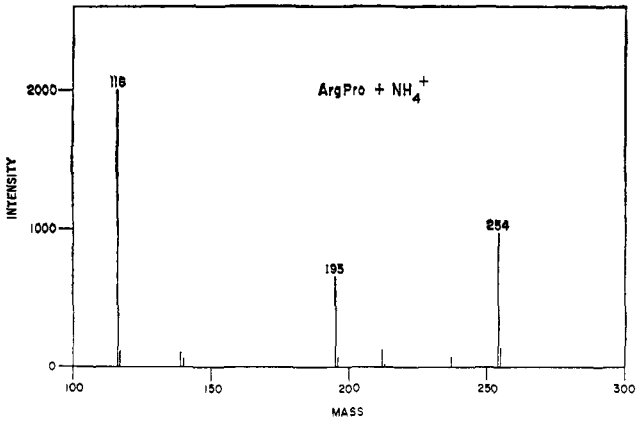


Figure 3. Proton transfer mass spectrum of Arg-Pro ionized with NH_4^+. The conditions are the same as given in the legend to Figure 2.

getics of the reaction favored by the stability of the products, protonated ornithine lactam and urea.

Spectra of N-Terminal Arginine Peptides. Spectra of Arg-Pro, Arg-Pro-Pro, Arg-Pro-Pro-Gly, and Arg-Pro-Pro-Gly-Phe are presented in Figures 3–6 and structural correlations are given in Table I. The significant features of the spectra shown are: (1) peptide chains are cleaved preferentially at peptide bonds, frequently with hydrogen atom rearrangements which produce stable neutral molecular species and protonated stable molecules; (2) probable processes include elimination of H_2O, NH_3, guanidine, and CO; (3) carboxyl terminal residues survive and are identified by their respective masses which provide a key to the correlation of spectra with amino acid sequence structures. A fragmentation scheme showing decomposition processes in the Arg-Pro spectrum is given below. Similar schemes for the other peptides studied can be readily constructed and will not be presented. The highest molecular weight species observed in the Arg-Pro spectrum is *m/e* 254 (II below). It is formed either by dehydration of the peptide prior to evaporation or by loss of water from the protonated parent molecule ion, *m/e* 272. Protonated C-terminal proline, I, is formed by breaking the Arg–Pro peptide bond and rearrangement of a hydrogen atom. No indication of the precise location of the ionizing proton is given. Attachment to the N atom is probable but not established. The hydrogen atom rearrangement leaves behind a neutral fragment which can isomerize into a ketene derivative. Here again the stability of neutral fragments can play a significant role in the energetics of the overall reaction. The rupture of the peptide bond and hydrogen atom rearrangement can take place on the probe surface with neutral dipeptide or with the protonated gaseous dipeptide ion. It is reasonable to assume that the protonated proline ion is not formed by decomposition of the protonated diketopiperazine, II. The three major ions in the Arg–Pro spectrum appeared with very similar time and hence temperature dependence during the heating of the sample probe. This observation suggests that they may all be derived from the same neutral gaseous species. However, the possibility of very similar rates of dehydration and peptide bond rupture on the surface cannot be ruled out. If this were the case and surface decomposition

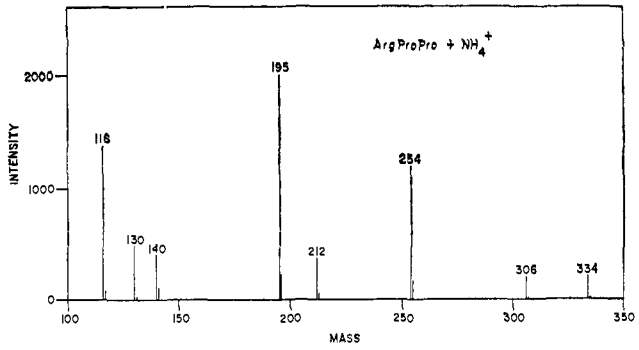
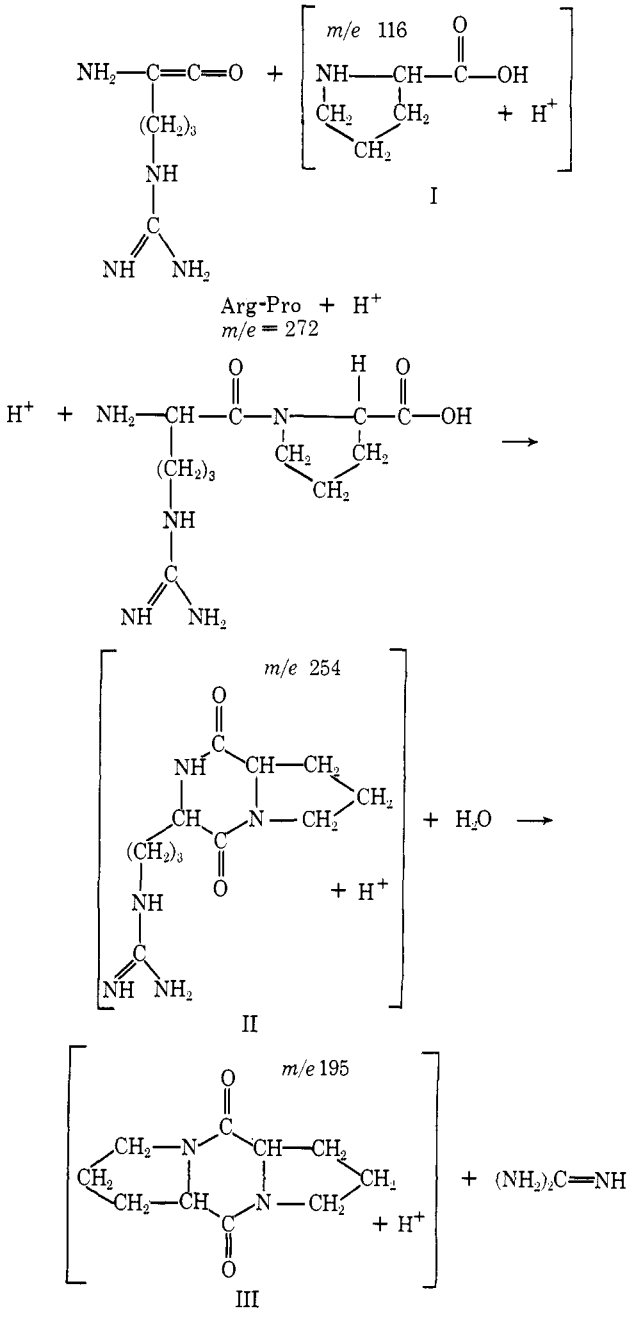


Figure 4. Proton transfer mass spectrum of Arg-Pro-Pro ionized with NH_4^+. The conditions are the same as given in the legend to Figure 2.



reactions rather than evaporation were rate-limiting processes then the observed time dependences of peak intensities would be obtained. Further studies with model compounds might clear up this point.

Table I. N-Terminal Arginine Peptides

Molecule	Mass	Fragment Arg-Pro-Pro-Gly-Phe	+[a]	−[a]
Arg-Pro	116	Pro	2H	
157 114[b]	195	Arg-Pro	H	$(NH_2)_2C{=}NH$, H_2O
	254	Arg-Pro	H	H_2O
Arg-Pro-Pro	116	Pro	2H	
157 97 114	130	Arg		HCN
	140	Arg		NH_3
	195	Pro-Pro	H	H_2O
		Arg-Pro		$(NH_2)_2C{=}NH$
	212	Arg-Pro		$NH_2C{\equiv}N$
	254	Arg-Pro		
	306	Arg-Pro-Pro		NH_3, HCOOH
	334	Arg-Pro-Pro		H_2O, NH_3
Arg-Pro-Pro-Gly	155	Pro-Gly	2H	H_2O
157 97 97 74	173	Pro-Gly	2H	
	195	Pro-Pro	H	
		Arg-Pro		$(NH_2)_2C{=}NH$
	254	Arg-Pro		
Arg-Pro-Pro-Gly-Phe	115	Arg		$NH_2C{\equiv}N$
157 97 97 57 164	120	Phe	2H	HCOOH
	139	Arg		H_2O
	155	Pro-Gly	H	
	166	Phe	2H	
	195	Pro-Pro	H	
		Arg-Pro		$(NH_2)_2C{=}NH$
	205	Gly-Phe	2H	H_2O
	212	Arg-Pro		$NH_2C{\equiv}N$
	223	Gly-Phe	2H	
	254	Arg-Pro		
	284	Pro-Gly-Phe	2H	$2H_2O$
	320	Pro-Gly-Phe	2H	
	333	Arg-Pro-Pro		H_2O

[a] + and − indicate atoms, molecules, or fragments gained or lost in the proton transfer ionization and decomposition processes. The + and − species are postulated to account for masses of the observations. [b] Masses of the respective N-terminal, C-terminal, and internal amino acid residues in the various peptides are given directly below these residues.

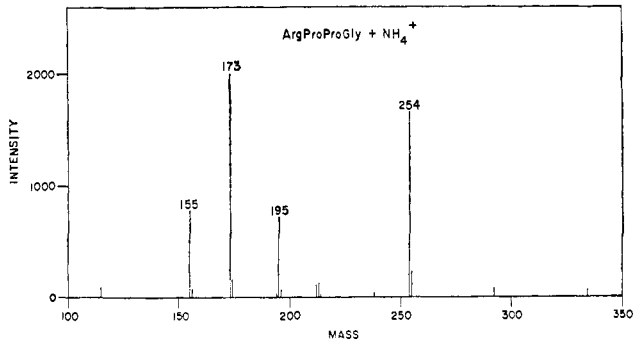


Figure 5. Proton transfer mass spectrum of Arg-Pro-Pro-Gly ionized with NH_4^+. The conditions are the same as given in the legend to Figure 2.

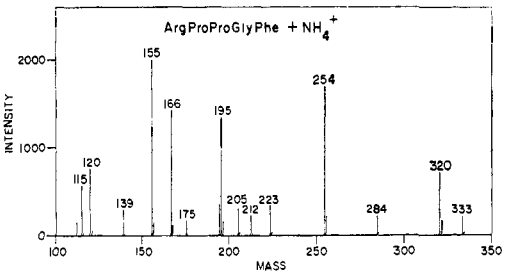


Figure 6. Proton transfer mass spectrum of Arg-Pro-Pro-Gly-Phe ionized with NH_4^+. The conditions are the same as given in the legend to Figure 2.

The only other ion found in appreciable yield in the Arg-Pro spectrum is *m/e* 195 (structure III). The formation of this ion having the mass of a protonated Pro-Pro diketopiperazine points up the need of an independent amino acid analysis of the peptide sample if spectra and structure are to be correlated. With an independently determined ratio of one arginine to one proline residue the observation of *m/e* 116 and *m/e* 254 establishes the dipeptide sequence in Arg–Pro.

Identification of the ions observed in the spectra of Arg-Pro-Pro, Arg-Pro-Pro-Gly, and Arg-Pro-Pro-Gly-Phe is made in Table I. The *m/e* 254 ion is common to all of the N-terminal Arg-Pro peptides studied here. This ion which has the mass of the protonated diketopiperazine is formed with a complementary stable neutral molecule (water, an amino acid, or peptide). The *m/e* 195 peak which is strong in the spectra of the peptides having the Pro-Pro sequence element has the mass of a protonated Pro-Pro diketopiperazine which is formed directly by rupture of peptide bonds or from the Arg-Pro ion by loss of guanidine. The *m/e* 155 Pro-Gly protonated diketopiperazine ion is found in the spectrum of Arg-Pro-Pro-Gly and Arg-Pro-Pro-Gly-Phe but the *m/e* 173 ion which has the mass of a protonated Pro-Gly dipeptide is found only in the Arg-Pro-Pro-Gly spectrum. The formation of this protonated dipeptide requires a hydrogen atom rearrangement, leaving behind a neutral fragment that can have the structure of a stable neutral molecule possibly a ketene derivative similar to the species suggested as the complementary neutral fragment formed with protonated proline in the Arg-Pro spectrum. The Pro-Gly dipeptide (*m/e* 173)

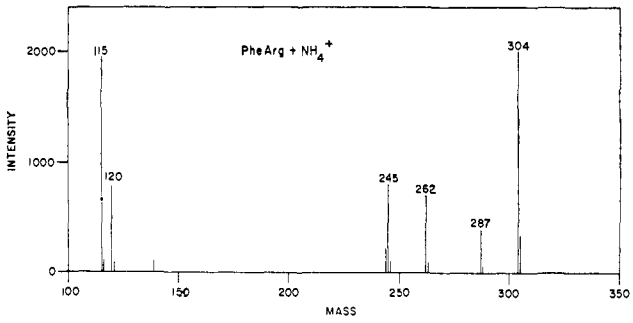


Figure 7. Proton transfer mass spectrum of Phe-Arg ionized with NH_4^+. The conditions are the same as given in the legend to Figure 2.

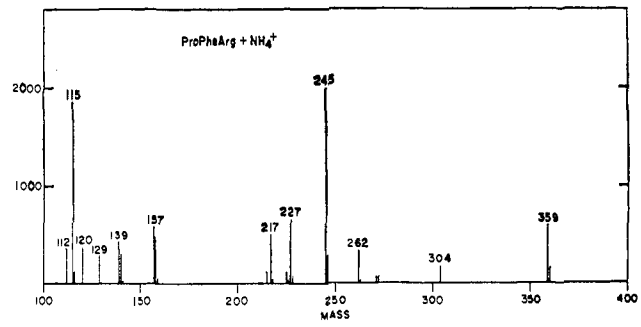


Figure 8. Proton transfer mass spectrum of Pro-Phe-Arg ionized with NH_4^+. The conditions are the same as given in the legend to Figure 2.

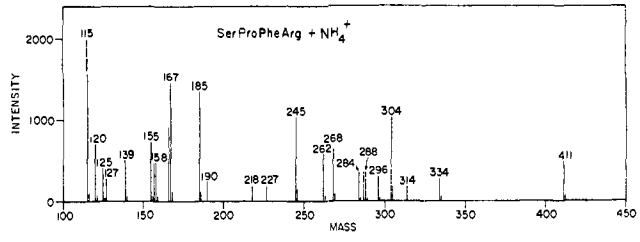


Figure 9. Proton transfer mass spectrum of Ser-Pro-Phe-Arg ionized with NH_4^+. The conditions are the same as given in the legend to Figure 2.

and carboxyl terminal glycine observed at *m/e* 76, but not shown in the spectrum given in Figure 4, distinguish Arg-Pro-Pro-Gly from the other N-terminal arginine peptides. Arg-Pro and Arg-Pro-Pro are distinguishable mainly in the higher mass regions, *m/e* 334 and *m/e* 306, ions formed by loss of H_2O, NH_3 and in the latter case NH_3 and HCOOH, from the protonated parent Arg-Pro-Pro ion.

Arg-Pro-Pro-Gly-Phe contains a strong peak corresponding to protonated phenylalanine, *m/e* 166, which establishes the carboxyl terminal amino acid residue. There is in addition the protonated Pro-Gly-Phe peptide at *m/e* 320. Both the *m/e* 166 and *m/e* 320 peaks are formed with hydrogen atom rearrangements.

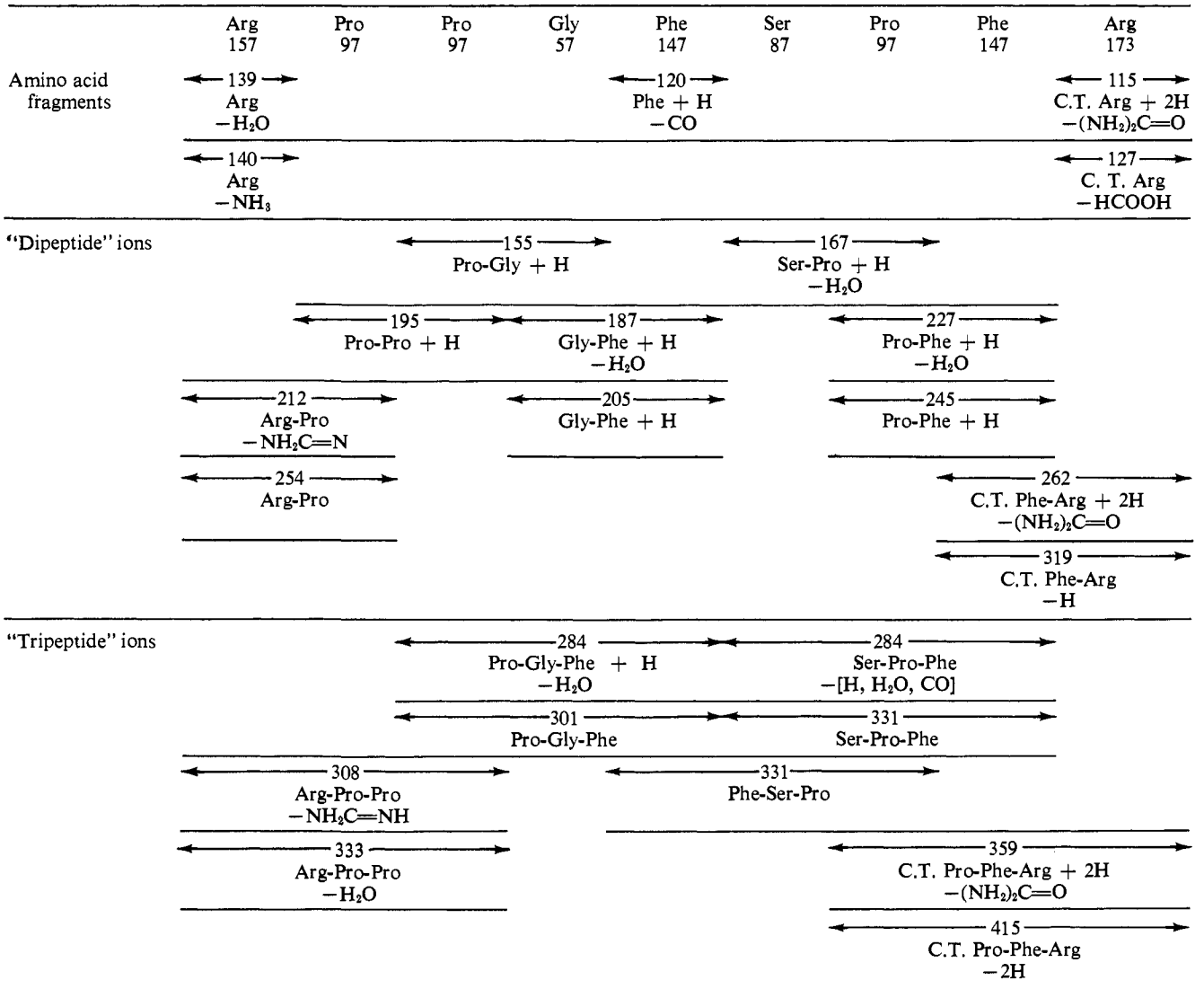
An unexpected peak of moderate intensity was found at *m/e* 115 in the Arg-Pro-Pro-Gly-Phe spectra. The fragmentation process responsible for this peak in the free amino acid arginine spectra has been discussed. It is possible that with the pentapeptide the carboxyl terminal group can be located in sufficiently close proximity to the N-terminal arginine guanidine moiety to permit lactam formation with the elimination of a urea molecule. The observation of an *m/e* 115 peak in one of four N-terminal arginine peptides, even in somewhat smaller abundance than with the C-terminal arginine peptides (see below), points up the hazards of structural correlations and the need for more extensive studies to establish this mass spectral approach to peptide structure determination. The use of the *m/e* 115 peak to identify C-terminal arginine residues requires the support of a search for alternative C-terminal residues. If none are observed then the assignment of *m/e* 115 to a C-terminal arginine gains credibility.

Spectra of Carboxyl Terminal Arginine Peptides. Mass spectra of Phe-Arg, Pro-Phe-Arg, and Ser-Pro-Phe-Arg are presented in Figures 7–9 and identification of masses is made in Table II. The *m/e* 115, 120, 245, 262, and 304 ions are found in all three spectra. The 304 peak corresponds to the mass of protonated Phe-Arg diketopiperazine ion. The *m/e* 262 is rationalized as a protonated phenylalanine ornithine lactam molecule ion, formed by elimination of urea from the protonated Phe-Arg dipeptide. The 245 peak was attributed to a protonated Pro-Phe diketopiperazine. Its presence in the Phe-Arg spectra would be a source of confusion in correlation of spectra with structure if the amino acid analysis of the peptide were not available and if the formation of a proline residue from arginine by loss of guanidine were not a familiar process. This type of ambiguity in correlation of structure with

Table II. C-Terminal Arginine Peptides

Molecule	Mass	Fragment Ser-Pro-Phe-Arg	+	−
Phe-Arg	115	Arg	2H	$(NH_2)_2C{=}O$
148 173	120	Phe		CO
	245	Phe-Arg	H	$(NH_2)_2C{=}NH$, H_2O
	262	Phe-Arg	H	$(NH_2)_2C{=}O$
	287	Phe-Arg	H	NH_3, H_2O
	304	Phe-Arg	H	H_2O
Pro-Phe-Arg	115	Arg	2H	$(NH_2)_2C{=}O$
98 147 173	120	Phe	H	CO
	129	Arg	2H	HCOOH
	139	Arg	2H	$2H_2O$
	157	Arg	2H	H_2O
	158	Arg	2H	NH_3
	217	Pro-Phe		CO
	227	Pro-Phe		H_2O
	245	Pro-Phe		
	262	Phe-Arg	2H	$(NH_2)_2C{=}O$
	304	Phe-Arg	2H	H_2O
	359	Pro-Phe-Arg	H	$(NH_2)_2C{=}O$
Ser-Pro-Phe-Arg	155	Ser-Pro		H_2CO
88 97 147 173	157	Arg	2H	H_2O
	158	Arg	2H	NH_3
	167	Ser-Pro		H_2O
	185	Ser-Pro		
	190	Ser-Pro-Phe		$C_6H_5CH_2$, CH_3OH, H_2O
	218	Pro-Phe	2H	CO
	227	Pro-Phe	H	H_2O
	245	Pro-Phe	H	
	262	Phe-Arg	2H	$(NH_2)_2C{=}O$
	268	Ser-Pro-Phe		$2H_2O$, CO
	284	Ser-Pro-Phe		H_2O, CO
	287	Phe-Arg	2H	H_2O, NH_3
	288	Phe-Arg	2H	$2NH_3$
	296	Ser-Pro-Phe		$2H_2O$
	304	Phe-Arg	2H	H_2O
	314	Ser-Pro-Phe		H_2O
	334	Ser-Pro-Phe	2H	
	411	Ser-Pro-Phe-Arg	H	$(NH_2)_2C{=}O$, NH_3, H_2O

Table III. Bradykinin Ion Fragments



spectra is a potential source of difficulty with several amino acid residues and constitutes a serious limitation in the application of this technique to peptide structural studies. The *m/e* 115 peak has been discussed in connection with the spectra of arginine and Arg-Pro-Pro-Gly-Phe. The *m/e* 120 peak is found in the spectrum of phenylalanine and phenylalanine containing peptides. It is formed by loss of CO from the protonated phenylalanine residue in the peptide chain.

The spectra of the C-terminal arginine peptides are constituted mainly of ions formed by loss of urea, water, ammonia, and carbon monoxide or combinations of these stable molecules along with ions produced by peptide bond rupture. The spectra correlate well with structure in that there is no evidence for C-terminal phenylalanine, proline, or serine nor is there evidence for combinations of dipeptides or their fragments which are not expected from the known sequences of the peptides.

The data presented may be considered as complementary to values obtained in the field desorption technique developed by Beckey and his collaborators[8] which has been used to obtain spectra of the methyl ester of the acetylated Gly-Arg-Arg-Gly peptide. The field desorption spectrum of this molecule gave an intense protonated parent molecule ion at *m/e* 501 and fragments at *m/e* 245 and 257 produced by cleavage of the Arg–Arg peptide bond. The experiment of Beckey and coworkers constitutes, to the best of our knowledge, the only example of a mass spectrum of a peptide containing two underivatized arginine residues. Results on our study of bradykinin which contains two underivatized arginine residues are presented below.

Correlation of Mass Spectra with Bradykinin Structure. The proton transfer mass spectrum produced by NH_4^+ ion impact on gaseous species obtained from rapid heating of small samples (~2 nmol) of bradykinin is given in Figure 10. The mass range presented is from *m/e* 100 to 600. The product ions are identified in Table III which is headed by the amino acid sequence of bradykinin with the masses of the respective amino acids listed directly below each element of the sequence. The table is divided into groups of ions based on the number of amino acid residues contributing to the mass of each ion. Since the lower end of the spectrum is cut off at *m/e* 100, the only "amino acid" peaks are derived from arginine or phenylalanine residues. Proline, glycine, and serine residues and the fragments of arginine and phenylalanine contribute relatively little to correlation of spectra with structure in a relatively large

Table III (*Continued*)

"Tetrapeptide" ions	371	Pro-Gly-Phe-Ser $-H_2O$
	371	Gly-Phe-Ser-Pro $-H_2O$
	389	Pro-Gly-Phe-Ser + H
	389	Gly-Phe-Ser-Pro + H
	390	Arg-Pro-Pro-Gly $-H_2O$
	398	Pro-Pro-Gly-Phe
"Pentapeptide" ions	494	Arg-Pro-Pro-Gly-Phe $-[NH_2C{=}NH, H_2O]$
	494	Arg-Pro-Pro-Gly-Phe $-[(NH_2)_2C{=}NH, H_2]$
"Hexapeptide" ions	584	Arg-Pro-Pro-Gly-Phe-Ser + H $-[(NH_2)_2C{=}NH, H_2]$
	584	Pro-Pro-Gly-Phe-Ser-Pro + 2H
	586	Pro-Gly-Phe-Ser-Pro-Phe $-[CO, H_2O]$
	587	Pro-Gly-Phe-Ser-Pro-Phe + H $-[CO, H_2O]$

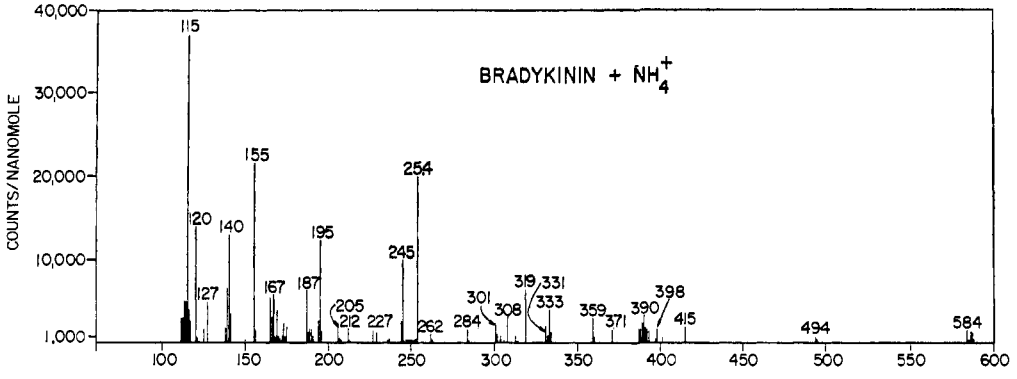
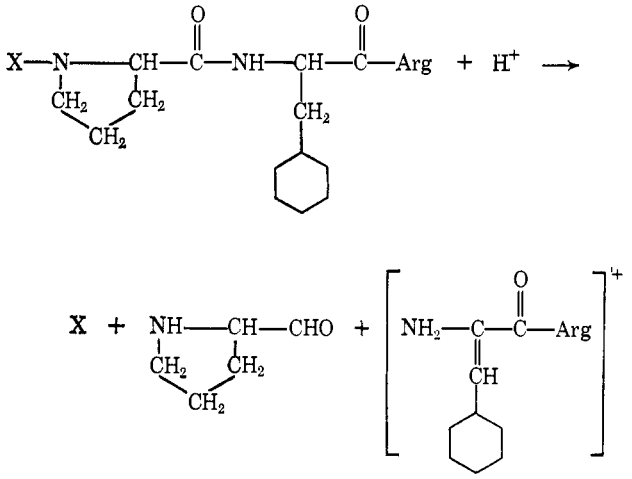


Figure 10. Proton transfer mass spectrum of bradykinin ionized with NH_4^+. In this composite spectrum the ordinate has been normalized to give the total integrated number of counts at each mass under the experimental condition of a primary beam intensity of 1×10^{-8} A. The experimental conditions are the same as given in the legend to Figure 2.

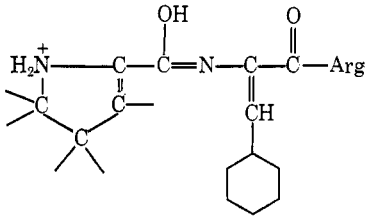
and complex proton transfer spectrum. Perhaps the most significant observation with respect to the spectra below *m/e* 100 is the failure to observe a protonated glycine molecule ion indicating that glycine is not located in a C-terminal position. The observation of *m/e* 115 and 127 peaks is consistent with C-terminal arginine. Ions above mass 600 in the bradykinin spectrum were of too low intensity to permit accurate mass identification with the available mass analyzer.

The "dipeptide" ions generally have the masses of protonated diketopiperazines and as seen from Table III overlap each other sufficiently to suggest the Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg sequence elements of the bradykinin spectrum. Phe-Ser is conspicuously absent as a dipeptide fragment. There is an ambiguity at *m/e* 195 not shown in Table III. The 195 peak as noted above should be listed as coming in part from the Arg-Pro dipeptide fragment as well as from Pro-Pro. The Phe-Arg fragments are noteworthy in that *m/e* 262 which is common to the C-terminal arginine peptides, Phe-Arg, Pro-Phe-Arg, and Ser-Pro-Phe-Arg, is a product of hydrogen atom rearrangement and urea elimination, a process similar to the one observed in the formation of *m/e* 115. The *m/e* 319 peak

can be an unsaturated dipeptide ion.[14b] A proposed fragmentation scheme for formation of *m/e* 319 is



The benzene ring in the phenylalanine residue is conjugated with the peptide carboxyl double bond and the resonance stabilization available in the conjugated unsaturated ion provides a driving force favoring hydrogen atoms rearranging out of rather than into the product ion. The formation of "hydrogen deficient" or unsaturated ions is noted with tri- and tetrapeptide residues containing phenylalanine, *e.g.*, *m/e* 284, 301, 331, 398, and 415 (*cf.* Table III). The *m/e* 415 ion (C. T. Pro-Phe-Arg) is a particularly interesting case in which two H atoms plus the ionizing proton have migrated out of the product ion leaving the following possible structure. Alternative peptide residue combina-



tions were considered for *m/e* 319 and 415 (limited to the available residues in bradykinin) and if bond rupture processes are limited initially to peptide bonds with possible subsequent elimination of the small stable molecules observed in the spectra of smaller arginine containing peptides, the mass assignment given appears to be unique. The tripeptide ions again show overlap of amino acid residues which support the Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg elements of the bradykinin sequence. The ambiguity between Ser-Pro-Phe and Phe-Ser-Pro sequence elements both having *m/e* 331 does not permit the use of this ion to establish the Phe-Ser linkage.

The tetrapeptide ions at *m/e* 371 and 389 can be correlated either with the Pro-Gly-Phe-Ser or Gly-Phe-Ser-Pro elements of the bradykinin sequence. In spite of the ambiguity these ions tie the Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg elements together uniquely.

Identification of many of the lower mass ions is supported by information provided in the spectra of smaller arginine containing peptides. The *m/e* 359 peak is found in the Pro-Phe-Arg spectrum; *m/e* 284 is found both in the Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg spectra and is assumed to be derived from either Pro-Gly-Phe or Ser-Pro-Phe by loss of water or water and CO, respectively, from the protonated tripeptide fragments. The *m/e* 333 ion identified with a water loss from the protonated Arg-Pro-Pro fragment is found in the Arg-Pro-Pro-Gly spectrum.

The "tetra-", "penta-", and "hexapeptide" fragments were assigned amino acid residues on a trial and error basis with no assistance from the model peptide spectra.

The identification of the tetrapeptide fragments with bradykinin sequence elements was relatively straightforward. The pentapeptide *m/e* 494 peak can be formed by loss of 61 mass units from the Arg-Pro-Pro-Gly-Phe fragment (*m/e* 555) in at least two ways. Formation of this ion from protonated Ser-Pro-Phe-Arg, *m/e* 505, would require an extremely improbable 11 mass unit loss. Decomposition reactions of other possible combinations of amino acid residues are equally improbable.

The hexapeptide ions presented similar problems of mass correlation with elements of the bradykinin sequence; *m/e* 584 can be formed from Arg-Pro-Pro-Gly-Phe-Ser by loss of guanidine from the protonated peptide fragment or it can be a Pro-Pro-Gly-Phe-Ser-Pro protonated species with an extra hydrogen atom. It is unlikely that this ion contains either C-terminal Arg or the C-terminal Phe-Arg residues. The 586 and 587 ions which are slightly less abundant are correlated with the Pro-Pro-Gly-Phe-Ser-Pro-Phe peptide residues with loss of CO from the phenylalanine and water for the *m/e* 586 and similar processes for the protonated species to give *m/e* 587.

The difficulty in correlation of spectra with structure with higher molecular weight fragments arises primarily because of problems of small molecule (water, CO, guanidine, etc.) loss and hydrogen atom rearrangements. The case for the correlations made would be significantly weaker without the spectra of the smaller peptides. However, if bradykinin were treated as an unknown, the spectra with an independent amino acid analysis would suffice to provide an extremely strong argument for the correct amino acid sequence. The basis for such an argument would be the overlap of sequence in the di-, tri-, and tetrapeptide fragments with the evidence for C-terminal arginine at *m/e* 115, 262, 319, and 415. The weakest point is the overlap at the Phe–Ser linkage. The *m/e* 371 and 389 peaks supported by the circumstantial evidence of *m/e* 331 and the 584, 586, and 587 peaks provide support for this element of the sequence.

On this basis a unique sequence for bradykinin which is consistent with all the available data can be constructed from the spectrum of the nonapeptide interpreted in terms of the spectra of the smaller peptides. Previous attempts to determine the complete amino acid sequence of arginine derivatized, permethylated bradykinin by mass spectrometry have been unsuccessful. Lenard and Gallop[15] determined the sequence of the four residues from the N terminus and three residues at the C terminus but could not locate Phe[5]-Ser[6]. Leclercq, Smith, and Desiderio[16] sequenced residues 1–7 of bradykinin but did not observe C-terminal sequence ions. A C-terminal dipeptide fragment ion (Phe,Arg) was identified which, however, could not be sequenced.

(15) J. Lenard and P. M. Gallop, *Anal. Biochem.*, **29**, 203 (1969).
(16) P. A. Leclereq, L. C. Smith, and D. M. Desiderio, Jr., *Biochem. Biophys. Res. Commun.*, **45**, 937 (1971).

An alternative to determining the sequence of bradykinin directly by mass spectrometry would be to subject the nonapeptide to partial acid hydrolysis in order to produce a limited number of small, more easily sequenced peptide fragments which also contain overlap information.[17,18] This approach is illustrated by the data presented in Table IV. The peptides in the partial

Table IV. Partial Acid Hydrolysis of Bradykinin

Peptide	Time at 110°, min	Peptide mol/mol Bradykinin 2 *N* HCl	4 *N* HCl	6 *N* HCl
Arg-Pro-Pro-Gly-Phe	5			0.25
	10	0.28	0.13	0.08
	15	0.25		
	20		0.03	
Arg-Pro-Pro-Gly	5			0.44
	10	0.40	0.45	0.51
	15	0.48		
	20		0.52	
Arg-Pro-Pro	5			0.02
	10	0.02	0.19	0.26
	15	0.17		
	20		0.04	
Arg-Pro	5	*a*		0.01
	10	*a*	0.09	0.12
	15	0.07		
	20		0.15	
Phe-Ser-Pro-Phe-Arg	5			0.12
	10	0.29	0.13	0.08
	15	0.25		
	20		0.04	
Ser-Pro-Phe-Arg	5			0.40
	10	0.27	0.30	0.30
	15	0.29		
	20		0.20	
Pro-Phe-Arg	5			*a*
	10	*a*	*a*	*a*
	15	*a*		
	20		0.04	
Phe-Arg	5			0.11
	10	0.42	0.12	0.20
	15	0.14		
	20		0.19	

[a] Less than 0.01 mol/mol of bradykinin.

acid hydrolysates were separated and identified with a peptide analyzer based on the amino acid analyzer. They were not examined mass spectrometrically. Free amino acids, neutral peptides, and basic peptides not listed in the tables were not determined. The overlap peptides, Arg-Pro-Pro-Gly-Phe and Phe-Ser-Pro-Phe-Arg, were produced in yields of approximately 25%. In general the cleavage pattern obtained illustrates the initial rather specific hydrolysis liberating the free amino group of the β-hydroxy amino acid serine. If a peptide of the size and complexity of bradykinin were an unknown, a rational approach to the problem of sequencing should combine direct mass spectrometry of the underivatized peptide, the use of partial acid hydrolysis, followed by mass spectrometry on the mixture and/or purified peptide fragments and enzymatic hydrolysis when appropriate.[19]

The ability to do mass spectrometry on underivatized arginine peptides deserves special comment. Chemical derivatization reactions can sometimes perturb peptides.[20] The isolation of products of derivatization reactions carried out on very small samples can frequently be an extremely difficult task. With peptides of unknown structure a considerable research effort may be required to develop a technique suitable for small samples because of complications associated with impurities and side reaction products.[3,21,22] These problems are by-passed with a technique that operates on underivatized peptides. Finally, an advantage inherent in the examination of unknown compounds aside from the difficulties in the derivatization procedure is that partially derivatized natural materials are directly observed.

Single ion impact tandem mass spectrometry using the principles of volatility enhancement and rapid heating provides a method to sequence small underivatized arginine peptides at the nanomole level. Larger peptides such as bradykinin can be done with more effort. We believe that the significant processes involved are a limited pyrolysis of the peptide on the sample probe and a gentle ionization technique which permits ionization and thus detection of the fragments with a minimum perturbation due to the ionization process itself. The volatility enhancement technique is, however, sensitive to inorganic impurities which must be removed prior to dispersal of peptide samples on the Teflon probe. If this condition of sample purity can be met, and peptides from tryptic hydrolysis of proteins are chromatographically isolated from one another, then the technique described here provides a highly sensitive rapid instrumental approach to amino acid sequence determination of peptides at the nanomole level.

The method described in this report is novel and has so far been subjected to test with only a limited number of peptides. Work is in progress to assess the generality of the method. With our limited experience, it cannot yet be considered a general method.

Acknowledgment. The authors express their appreciation to Schwarz Mann, Orangeburg, New York, a Division of Becton, Dickinson and Co., for making available the synthetic Thyrotropin Releasing Hormone and to Dr. J. M. Stewart of University of Colorado Medical Center, Denver, for gifts of arginine-containing peptides.

(17) F. Sanger, *Advan. Protein Chem.*, 7, 1 (1952).
(18) R. L. Hill, *Advan. Protein Chem.*, 20, 37 (1965).
(19) M. M. Shemyakin, Y. A. Ovchinnikov, E. I. Vinogradova, A. A. Kirynshkin, M. Y. Feigina, N. A. Aldanova, Y. B. Alakhov, B. M. Lipkin, A. I. Miroshnikov, B. V. Rosinov, and S. A. Kazaryan, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 7, 8 (1970).
(20) R. Hodges, S. B. H. Kent, and B. C. Richardson, *Biochim. Biophys. Acta*, 257, 54 (1972).
(21) H. R. Morris, R. J. Dickinson, and D. W. Williams, *Biochem. Biophys. Res. Commun.*, 51, 247 (1973).
(22) H. M. Fales, G. W. A. Milne, J. J. Pisano, H. B. Brewer, Jr., M. S. Blum, J. G. MacConnell, J. Brand, and N. Law, *Rec. Progr. Horm. Res.*, 28, 591 (1972); H. M. Fales, *ibid.*, 28, 625 (1972).