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High-Mass Ion Fragmentation as a Function of Time and Mass

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The effects of molecular weight and ion lifetimes on the extent of fragmentation observed in mass spectra of compounds in the mass range 500–5800 have been studied. Fragmentation of large ions over long periods of time can be observed as coherent peaks in time-of-flight spectra when delayed ion extraction techniques are employed. The implications for the design of future high-mass instruments are discussed.

Several recent developments in mass spectrometry have expanded considerably the range of (bio)organic compounds accessible for study. The introduction of new techniques for ionization of involatile and thermally labile compounds, e.g. plasma desorption (PD) (*1*), fast (keV) atom (ion) bombardment (FAB) (*2*), and laser desorption (LD) (*3*), have allowed generation of gas-phase ions with masses as high as 23 000 daltons. In turn, this progress in desorption has stimulated development of mass analyzer and detector systems capable of handling ions above 3000 daltons (*4*, *5*). Initial studies by mass spectroscopists in the mass range above 3000 daltons have indicated that some of the concepts evolved historically for the mass range below 1000 have to be reexamined for the higher mass range.

It has been shown both theoretically and experimentally (*6*, *7*) that isotopic contributions to the envelopes of molecular ions in the range 3000 to 23 000 significantly reduce the ion currents carried by the familiar monoisotopic ions, broaden the envelope widths at half height beyond 10 atomic mass units, and encourage exploitation of average mass measurements even on instruments for which unit mass resolution is possible.

Secondly, while it has been suggested that the protonated species in desorption spectra are formed by ion/molecule reactions similar to those gas-phase processes of chemical ionization (*8*), this mechanistic explanation cannot necessarily be extrapolated to very large ions. Bovine insulin samples produce a distribution of singly and multiply charged molecular ions, which reflect their relative abundances in solutions of varying pH (*9*), suggesting that the desorption of preformed ions may become dominant at high mass and that the pK_a and isoelectric point are more important than ionization potential and/or gas-phase basicities for determining ionization efficiencies for large molecules.

High-mass spectra also appear to differ from low-mass spectra in the nature and extent of fragmentation. In particular, while small peptides such as leu-enkephalin (MW 556) yield complete N- and C-terminal sequence ions, the spectra of the insulins and proinsulins yield mainly molecular ions, nonspecific losses of small neutral groups contributing to the extended envelopes on the low-mass side of molecular ion envelopes (*10*, *11*), and a continuous background of incoherent fragment ions. The intensity of this background, which is observed in both sector magnet and time-of-flight (TOF) analyzers, decreases exponentially with increasing mass and drops abruptly (though not to zero) at the high-mass end of the molecular ion envelope. This incoherent fragmentation,

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chemical noise, or "peak at every mass" results in large part from homolytic cleavages and radical recombination reactions close to the site of primary particle impact on matrix and sample and is overlaid in many TOF instruments by metastable ions.

Several explanation for the absence of coherent fragmentation have been advanced. Barber et al. (*2*) suggest that the fragment ions are simply not intense enough and are lost in the incoherent noise. Macfarlane (*12*) has expressed the view that there is little or no fragmentation, while Chait has emphasized that fragmentation is extensive in large ions, but leads predominantly to low-mass and incoherent ions (*13*). The quasi-equilibrium theory (QET) has also been applied by several groups (*14–16*) to explain the fragmentation behavior of large molecules. For a particular internal energy the increase in the number of degrees of freedom in a large molecule results in a reduction of the rate constant of a particular fragmentation pathway. Bunker and Wang (*14*) have carried out a theoretical analysis of the unimolecular behavior of large chain-type molecules, which takes into account the variations in the frequency factor for bonds in different positions in the molecule. Their calculations predict preferential loss of small groups from the ends rather than cleavages in the center of the molecule.

In this study we attempt to demonstrate that fragmentation of large molecules does occur but is likely to be extended over very broad time periods, reflecting the lower rates for unimolecular decomposition of molecules with a very large number of degrees of freedom. This idea is tested by sampling ions (using time-of-flight mass analysis) at increasingly longer times after their formation. This is accomplished by increasing their source residence times (up to 1.7 μs on a PDMS instrument) by using lower accelerating voltages or (up to 13 μs on a liquid secondary ion mass spectrometry time of flight instrument) employing delayed ion extraction. Moreover, in the latter instrument we attempt to demonstrate that, in the longer time frame, one can observe analytically useful ions, i.e. sequence ions resulting from cleavages in the center of the molecule.

EXPERIMENTAL SECTION

Two mass spectrometric TOF systems equipped with different types of ionization sources have been used. One is a commercial ^{252}Cf PDMS (plasma desorption mas spectrometer) instrument (BIO ION Nordic, Sweden) interfaced to a PDP 11/73 based data acquisition and processing system. A constant voltage, or static field, is imposed across the ion acceleration region. The other is a modified CVC 2000 (Rochester, NY) TOF mass spectrometer, which employs a drawout pulse (i.e. dynamic TOF) for ion extraction. Primary ions (5 keV Xe^+) were provided by a Kratos (Ramsey, NJ) minibeam (high flux) ion gun. A LeCroy 3500 SA (Spring Valley, NY) system is used for high-speed data acquisition. The design and performance of that instrument are described in detail elsewhere (*17*).

The peptides leu-enkephalin (average MW 556), oxytocin (average MW 1007), melittin (average MW 2846), and crystalline bovine insulin (average MW 5733) were obtained from Sigma (St. Louis, MO) and used without further purification. For PDMS mass spectrometry 20 nM of each peptide, dissolved in acetic acid, was electrosprayed onto an Al foil. Each spectrum was accumulated through 1 000 000 primary events, with accelerating voltages ranging between 2 and 24 kV, corresponding to source residence times for melittin of 1.7, 1.2, 0.84, 0.69, 0.60, 0.53, and 0.48 μs, and 8–20 kV, corresponding to 1.2, 0.98, 0.85, and 0.76 μs for insulin.

The ion drawout pulse on the SIMS instrument allows source residence times to be varied without changing secondary ion kinetic energy. The peptide samples were dissolved in 0.1 M HCl, mixed with glycerol and applied to the probe tip. The sample size was approximately 10 μg. The primary ion beam of 5-keV Xe ions was pulsed at a rate of 100 Hz with a pulse width of 4 μs. The secondary ions are formed in the field-free source region and are extracted by a 150-V pulse from 2 to 20 μs after the primary ion pulse, accelerated through 3 kV, and analyzed by a 1-m field-free drift tube. After mass separation the ions are further accelerated to 12 keV and detected by Galileo (Sturbridge, MA) dual channel plates.

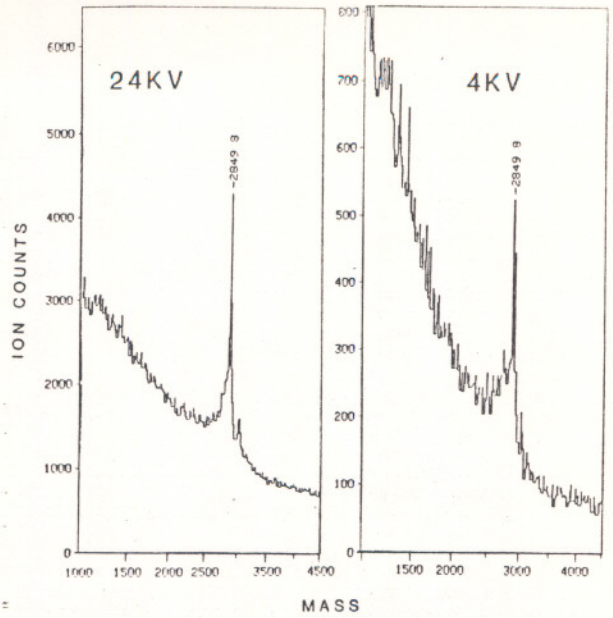

Figure 1. Plasma desorption mass spectra of melittin, obtained at source residence times of 0.48 and 1.19 μs, respectively. (Reprinted with permission from ref 19. Copyright 1986 John Wiley & Sons, Ltd.)

RESULTS AND DISCUSSION

It should be noted that the two instrumental configurations provide quite different ways to observe the degree of fragmentation. In the PDMS instrument, ion formation, extraction, and acceleration all occur in the same region. Since fragmentation during extended source residence time occurs in an electric field, fragment ions will be only partially accelerated and the extent of fragmentation must be measured as the increase in the slope of the incoherent background. In addition, lowering the accelerating voltage decreases both the transmission of the analyzer and the detector collection efficiency, both of which may discriminate against ions of high mass. These effects are minimized by integrating the ion current beginning at about half the mass of the molecular ion and comparing this with the molecular ion current. In the dynamic TOF, fragmentation occurs in a field-free source, so that fragment ions receive full acceleration and appear as peaks at their expected flight times and the dependence of fragmentation on residence time is more appropriately measured by comparing particular fragment ion intensities with the molecular ion intensity.

PDMS spectra of melittin obtained with accelerating voltages of 4 and 24 kV (corresponding to source residence times of 1.2 and 0.48 μs, respectively) are shown in Figure 1. Overall, fragmentation is observed to increase at longer ion lifetimes. Figure 2 presents the percent of the total ion counts in the mass range 1400–2850 carried by the protonated molecular ion envelope of melittin as a function of source residence time. This figure also compares results for leu-enkephalin (MW 556) and bovine insulin (MW 5733). As the mass of the molecular ion is increased, the increase in source residence time is seen to have a greater effect on the fragmentation.

The experiments carried out on the instrument with delayed drawout pulse allowed the study to be extended into the microsecond range and also provide the opportunity to carry out the experiment at constant accelerating voltage. A spectrum of melittin obtained on this instrument 13 μs after

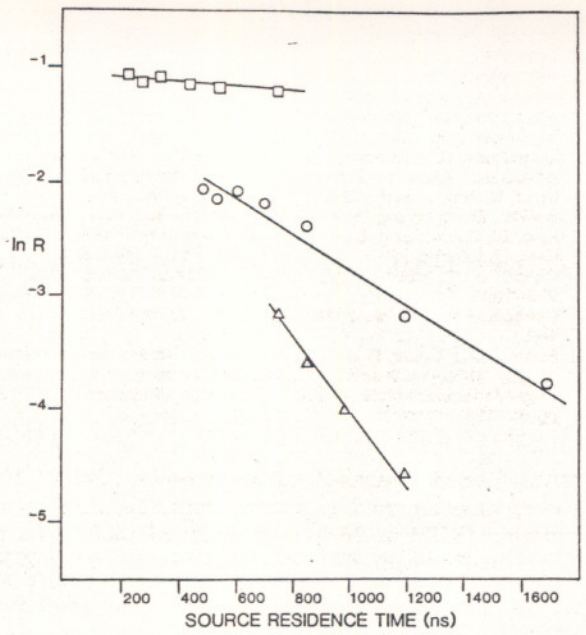

Figure 2. Ratio of molecular ion counts/total ion counts (in the range M/2 to M) in the plasma desorption mass spectra of leu-enkephalin (□), melittin (○) and insulin (△) as a function of source residence time.

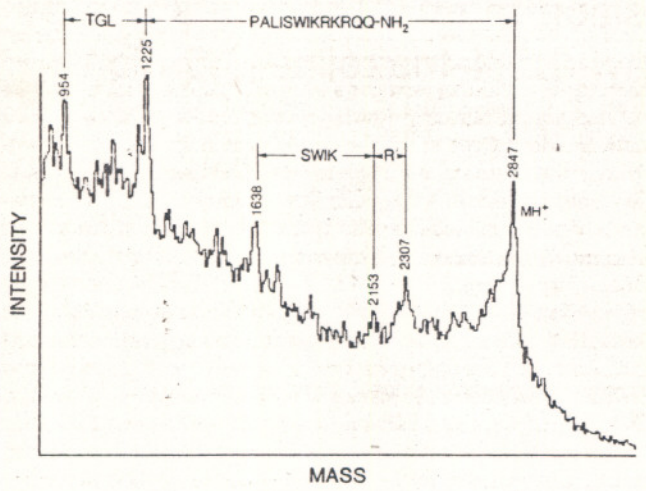

Figure 3. Liquid SIMS spectrum of melittin, obtained on the dynamic TOF, with the ion drawout pulse set 13 μs after the ionization pulse.

the primary ion pulse is shown in Figure 3. Specific fragment ions at 954, 1225, 1638, 2153, and 2307 amu, which correspond to cleavages in the peptide backbone, can be observed. In Figure 4 the ratios of the abundance of protonated molecular ions of melittin to masses 2153 and 2307 fragment ions are plotted as a function of the source residence time. The molecular to fragment ion ratio decreases with time. For comparison, the same determination was made for the peptide oxytocin (MW 1007), which shows a major cleavage at the proline bond (*17*). In this case fragmentation shows a slight decrease with residence time, which results from some discrimination at long residence times against ions of low mass, which, having higher velocities, will tend to collide with the source walls sooner than ions of larger mass. Here as in the PDMS experiment, it is the larger mass ions that appear to require longer times to undergo fragmentation.

It should of course be noted that two different ionization techniques are employed in these measurements. It is reasonable to expect that these techniques may impart different amounts of internal energy to desorbed molecules. In addition, bimolecular processes may be involved in ion formation, internal energy stabilization, and induction of fragmentation,

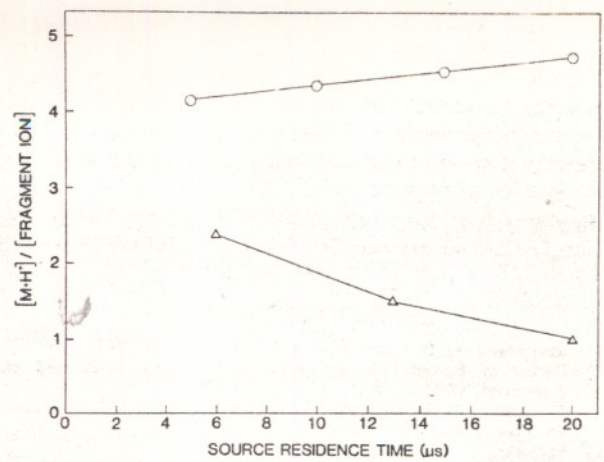

Figure 4. Ratio of the molecular ion/fragment ion intensity in the liquid SIMS mass spectra of melittin (△), fragments *m/z* 2153 and 2307, and oxytocin (○), fragment *m/z* 723, as a function of the delay time between the ionization pulse and the ion drawout pulse.

particularly through collisions close to the liquid/vacuum interface in the liquid SIMS technique. In that technique, ions are sampled by the analyzer at some distance (0.5–1.0 cm) from the surface, so that further bimolecular events can be ruled out as contributors to the time-dependent fragmentation behavior. Despite the differences in the mechanisms of ion formation, the general fragmentation behavior in both instruments with respect to time and mass of the ion is consistent.

CONCLUSIONS

An important observation on the fragmentation behavior of large molecules is that their lower unimolecular dissociation rates shift most of the fragmentation into the metastable time frame, defined operationally as dissociation after partial or full acceleration. For example, Chait and Field (*18*) have reported that molecular ions of insulin, formed by plasma desorption, are stable for long periods of time and continue to dissociate during the entire flight time from source to detector in a time-of-flight mass spectrometer. The experiments reported here, under configurations where ion lifetimes are extended *before* acceleration, confirm that fragmentation takes place at least through the range of 0.4–20 μs.

In addition, the observations reported here compare the fragmentation time frame for ions of different masses, extend the time frame by using the liquid SIMS instrument, and demonstrate that at least some of the fragmentation results in structurally informative ions. This has some important consequences analytically, particularly with regard to the present and future design of instruments intended for the analysis of high molecular weight compounds. In the PDMS instrument, even with accelerating voltages of 20 kV, a large ion spends a considerable time in the source/accelerating region (760 ns for bovine insulin). As a result, a considerable amount of metastable fragmentation occurs and may actually obscure prompt fragmentation. Thus, while we have used reduced acceleration voltages to probe the time frame, this is not an analytical solution, as delaying ions in this fashion increases incoherent low-mass signal, reduces resolution, and decreases our capabilities for distinguishing the molecular ion. Our experience with the dynamic TOF suggests that, for those instruments that base mass separation on ion energy, i.e. sector and TOF instruments, physical separation of source and accelerating regions combined with ion trapping techniques may improve our observation of fragment ions. We suggest that quadrupole ion storage traps, QUISTORs (used in combination with a mass analyzer or as analyzers themselves), and ICR (ion cyclotron resonance) instruments may play an important

role in the future, as compatible ionization techniques are developed. In any case, it is clear that large ions are not easily dealt with in the same fashion as smaller ions, and despite the instant successes that resulted from the addition of FAB to sector instruments, radically different approaches may be necessary if we are to be able to measure the ions that we are now capable of making.

Registry No. Xe^+, 24203-25-6; ^{252}Cf, 13981-17-4; leu-enkephalin, 58822-25-6; oxytocin, 50-56-6; melittin, 20449-79-0; insulin, 9004-10-8.

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Location of Conjugated Diene Position in an Aliphatic Chain by Mass Spectrometry of the 4-Phenyl-1,2,4-triazoline-3,5-dione Adduct

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The electrophilic reagent 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) is used to form Diels–Alder adducts with conjugated dienes. The reaction is rapid, selectively leading to cyclic Diels–Alder adducts with conjugated dienes, and takes place at room temperature. Formation of the cycloadducts can be readily recognized from their mass spectra. Their electron impact mass spectra are marked for their simplicity. In addition to displaying prominent molecular ion peaks, the spectra exhibit characteristic fragments, which are diagnostic of the position of the conjugated diene in the chain.

The determination of double bond position in a carbon chain has been a topic of interest for a number of years. Double bonds occur in a number of natural compounds, and conjugated dienes, in particular, are important in certain insects as pheromones, semiochemicals, and sex attractants (*1–5*).

Mass spectrometry is an attractive means of examining such compounds because of its high sensitivity and structural specificity. However, under normal electron impact (EI) ionization conditions the double bond can migrate prior to fragmentation yielding spectra that are difficult to interpret with respect to its original position. Approaches to this problem generally involve either soft ionization techniques such as chemical ionization (CI) or chemical modification of the olefin followed by conventional EI ionization. There are a number of recent examples of the former approach. Chemical ionization using Fe^+ reagent ions followed by collisional activation leads to fragment ions that are diagnostic of double bond position. This approach has been applied to both dienes and monoolefins (*6*). Other CI reagents that interact specifically with double bonds include methyl vinyl ether (*7*) and $Ar/O_3/H_2O$ mixtures (*8*). Isobutane CI mass spectrometry of conjugated dienes yields abundant diagnostic fragment ions (*9*). Field ionization conditions produce spectra in which double bond migration is not observed and in which allylic cleavages result in major ions that are indicative of the position of the double bond (*10*). Some of these methods, which require a relatively sophisticated instrumental approach, may not always be available to the general chemical practitioner

Methods involving electron impact ionization usually require chemical modifiction of the double bond to form derivatives that direct the fragmentation around it in order to indicate its position. Two well-known examples involve the conversion of the olefin to a glycol followed by either tri-

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