Biomolecules in the Gas Phase: Multiphoton Ionization Mass Spectrometry

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Introduction

Much of the presently available information about the nature of molecular systems and the properties of molecules in particular comes from experiments in the gas phase. Only in the gas phase are molecules not affected by solvent or environmental effects. Optical spectroscopy of all forms has contributed heavily to our knowledge of gas-phase molecular properties. This includes much of our understanding of energy levels, structure of molecules, reactivity, and photochemistry; in short, much of both structure and dynamics. This is due to the fact that there is no more unmistakable identification than that based on the investigation of isolated molecules. Therefore, gas-phase techniques are highly significant, not only for basic research studies but also for everyday analytics.

The introduction of molecules into the gas phase has been the primary limitation for such detailed techniques, and therefore, experiments undertaken have involved rather small molecules that either exist in the vapor phase or can be vaporized readily without thermal damage. Of the 7 million chemical compounds known, these criteria unfortunately only apply to a very small number, thus limiting us to extrapolating our chemical insight from information gleaned only from quite small systems—a long way from biomolecules. Biomolecules, however, belong to the very important class of compounds generally having negligible vapor pressure. Furthermore, heating such systems generally results in severe decomposition.

One of the most important new developments in the area of low-molecular-weight gas-phase experiments has been the expansion into molecular beams, an experimental capability that not only defines directionality and provides velocity selection but, in the case of pulsed-jet expansions, also yields a low-temperature environment down to a few kelvins even though the molecule is in the gas phase. Such an environment becomes essential to the spectroscopy of very large molecules as otherwise the number of possible bands becomes extremely large.

In this Account we describe a technique that enables the vaporization of biomolecules intact, as neutral species and without destruction, while introducing them into a molecular beam in a low-temperature environment.

In the analytical field of gas-phase methods, mass spectrometry is the most common technique used to yield in one step (1) molecular-weight information by an exact measurement of the molecular ion and (2) specific structure information through the fragmentation pattern. During the last decade, a number of novel ionization methods have been developed, particularly for labile compounds involving direct ionization. Although these techniques have some success, if ionization is soft, they are often fraught with extreme fragmentation due to the large amount of energy necessary for the simultaneous desorption and ionization. This results in a number of reactions during and after this initial step, thus preventing an easy interpretation of the resulting spectra.

Here we discuss a new technique we call LEIM–MUPI (laser evaporation of intact molecules–multiphoton ionization), which offers a number of advantages and yields intense, clear spectra with an extremely low background. This is due to subtle consequences resulting from the simple fragmentation mechanism which is characteristic of the MUPI technique. This method derives its advantage from the introduction of tunable lasers into these techniques. There are two main features that make the combination of LEIM–MUPI of importance in mass spectrometry: (1) desorption and ionization are fully separated in time and space and (2) the degree of fragmentation is controlled via the excitation energy (i.e., tunability of the dye laser). The latter capability makes it feasible to determine the degree of fragmentation, including the formation of only molecular ions. At this time we have realized only the combination of LEIM–MUPI with mass spectrometry, but this new technique also opens other possible ways of gas-phase spectroscopy to be applied to delicate biochemical samples, e.g., laser-induced fluorescence (LIF), emission spectroscopy, or photodissociation methods.

Vaporization of Molecules

During the last two decades, different attempts have been made to vaporize larger molecules into the gas phase. Simple thermal heating of substances can only be applied to smaller molecules with some thermal stability, while large molecules such as biomolecules will immediately decompose. Mass spectrometry particularly has developed a large number of methods for vaporizing thermally labile molecules, including fast atom

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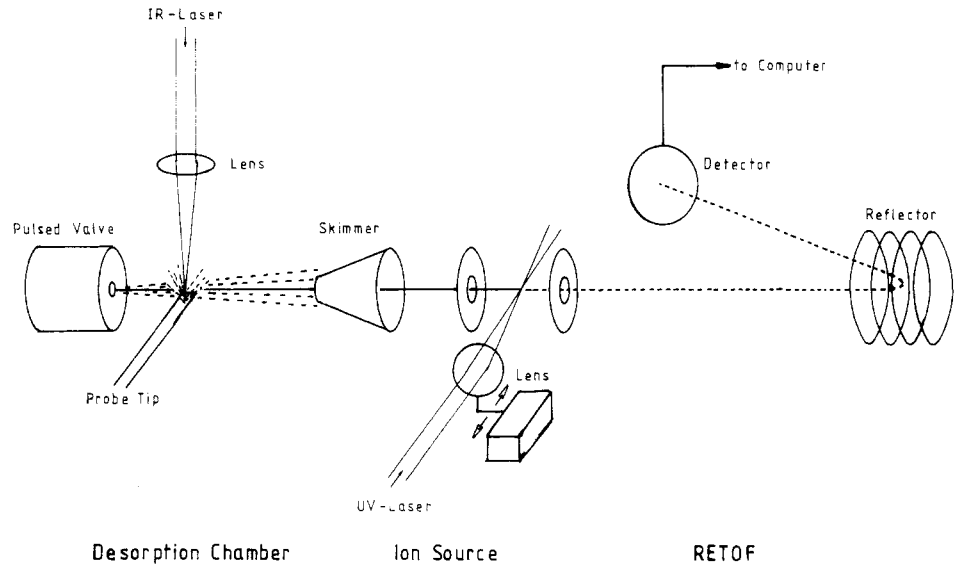

Figure 1. Principal scheme of the laser desorption–multiphoton ionization mass spectrometer.

bombardment (FAB),[1] secondary ion mass spectrometry (SIMS),[2] californium-252 plasma desorption (^{252}Cf-PD),[3] direct laser desorption (LD),[4-6] and electrospray vaporization.[7] It should be noted that all of these methods produce ions from very large biomolecules directly in the initial step. Especially laser desorption has shown this recently.[8] The parallel formation of neutral molecules by these methods generally has not been investigated. Some work, though, has been done to investigate the yield of all neutral molecules evaporated from a surface by these different methods.[9-13] Starting from the pioneering work by Kistemaker et al.[5] on biological molecules, direct laser desorption of ions became a valuable method for producing ions in a mass spectrometer. Depending on the laser wavelength, power, and experimental details, this ionic desorption process has been extensively investigated. In these investigations, high-powdered, pulsed CO_2 lasers[14] and Nd:YAG lasers[15] are used to induce a rapid heating that produces ions from a surface while suppressing decomposition as much as possible.[11-20]

Cotter and co-workers[19] have shown that laser desorption produces not only ions but also neutrals with a much larger yield than ions. Furthermore, they concluded that the formation of neutrals and the formation of ions are two separate and independent processes. Prompt ions were detected during the first few microseconds after the laser pulse, but the neutral molecule production process continued for about 100 μs. The desorbed neutral species were detected after ionization with a pulsed electron beam. The actual number of neutrals compared to the ions formed during the laser pulse was several orders of magnitude larger (typically 10^4:1) for power densities below 10^8 W/cm^2 in the absence of salts on the surface.

Making use of this very strong desorption of neutrals opens up the possibility of using postionization techniques, perhaps even more effectively than the one-step procedure. In 1982, it was shown by Selzle et al.[21,22] that desorbing neutrals with a low-powdered IR laser and forming ions with multiphoton excitation produces soft-ionization mass spectra. These results indicate that these spectra evolve from intact neutral molecules. Recently, several advances have been made, including the incorporation of a Mamyrin-type reflectron time-of-flight spectrometer,[23-25] the use of a UV laser for the

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desorption of molecules from surfaces,[26] and postionization techniques for neutral species desorption.[27-29]

In our experimental setup[30] (Figure 1), the desorption laser (either a CO_2 (10.6 μm) for a Nd:YAG (1.06 μm) laser) is focused to a 1–2-mm^2 spot on a metallic sample holder on which the sample is distributed either neat or as part of a matrix. The power density on the sample holder is about 10^4–10^5 W/cm^2 or 2–3 orders of magnitude less than used in the typical direct laser desorption experiments.[19] Due to this low power density, no ions are formed in the desorption process,[31] although thermal decomposition can still be a problem in some cases unless special precautions are taken.

The experimental conditions associated with laser desorption of neutrals has been modeled theoretically.[14,20] As a result of this analysis, the desorption of neutral molecules by the impact of IR photons has been attributed to a purely thermal effect. Recent data obtained in our group,[32] however, show that the desorption of neutral molecules is not solely thermal but that also nonthermal processes play an important role. Evidence is accumulating that multilayer samples lead to hyperthermal emission, which is possibly associated with macroscopic fissures in the solid sample.

The desorption is performed just after the orifice of a pulsed valve, and hence desorbed molecules are seeded in the noble gas before the expansion takes place. The specific characteristics of the supersonic beam formed by an isentropic expansion of noble gas molecules, therefore, are important. Since the detailed features of supersonic beams in spectroscopy and chemistry have been reviewed recently,[33,34] a description of these aspects is not given here, but rather the unique characteristics of the postionization procedure which distinguishes our experimental approach is discussed.

The cooling effect arises from a large pressure drop after the orifice of the nozzle. Since the diameter of this orifice is smaller than the mean free path of the molecules, a large number of collisions between the molecules will occur. These collisions yield a large velocity component in the axial and forward direction of the mass flow. As a result, a much narrower velocity distribution is produced than at room temperature at a much-enhanced on-axis density. Therefore, the thermal motion of the molecules expanded into the vacuum is converted into the directed mass flow of the jet and cooling of the different degrees of freedom of the molecules occurs. Any ions produced are deflected, due to the experimental setup. As a result, the desorption and the chosen postionization process are completely separated in time and space.

Multiphoton Ionization

Multiphoton mass spectrometry relies on lasers to tune to a resonant state as an intermediate step on the way to ionization with a further photon.[35] This is essential as it not only assures selectivity of the ionization events but also greatly reduces the intensity required for ionization. If the light is not resonant with an absorbing molecular level, absorption of light can still occur via virtual states, but at much higher levels of intensities. At high enough laser intensities, everything ionizes, even the background gas in the ion source. Once this nondiscriminant excitation reaches ionization, extreme fragmentation usually results; i.e., once ions are formed, they readily absorb more photons to produce severe fragmentation and even total atomization. Such fixed-frequency ionization[36,37] and mass selection was carried out before tunability of the laser source was discovered to be an essential feature to produce useful mass spectra particularly in terms of the extreme selectivity and sensitivity. Mass-selected absorption spectra obtained by a laser have been reported from sodium and potassium in 1977.[38] Mass spectra of organic molecules with tunable light sources were discussed by Letokhov et al.[39] and produced in 1978 by Boesl et al.[40] and Zandee et al.[41] In particular, mechanistic processes provided a number of unique features that have contributed to the utility of this method. In the last decade, a very large number of papers appeared and multiphoton ionization has been reviewed extensively in its physical properties.[42-47]

It should be emphasized that a multiphoton excitation can only result in selective ion formation if (1) the molecule has a chromophoric group to accept the photons, (2) the energy sum of the photons exceeds the ionization energy of the molecule, and (3) the molecule has an intermediate electronic state with a lifetime long enough not to substantially decompose during the pumping process. As indicated in Figure 2, the fluorescence decay of the intermediate electronic state, the intersystem crossing from the singlet to a triplet electronic state, and the neutral decomposition of the electronic activated state are possible side reactions, but through proper steering of the absorption process, their influence on the spectra can be suppressed.

One of the peculiar and very useful features of

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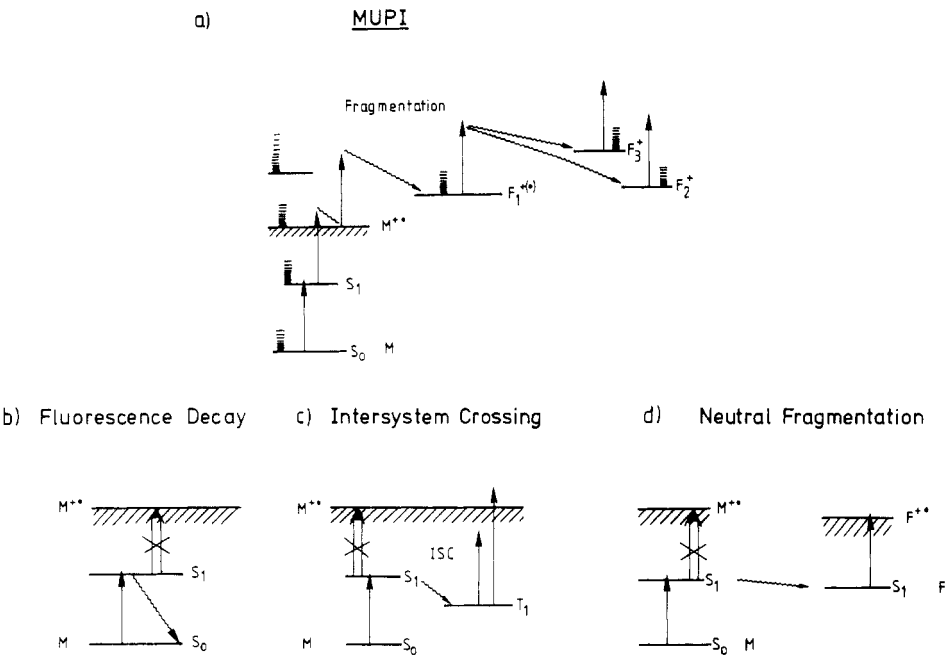

Figure 2. (a) Multiphoton-ionization process with subsequent fragmentation induced by further absorption of photons in the molecular ion or fragment ions. (b) Absorption of a photon leads to an excited intermediate state, but fluorescence prevents ionization. (c) The intermediate excited state S_1 undergoes an intersystem crossing to a triplet state, which cannot be ionized by a photon of the same wavelength used for activation. (d) The excited state underwent a fragmentation reaction, thus forming a new molecule, thus preventing observation of the starting molecule.

multiphoton excitation is the stepwise activation of a molecule (Figure 2). Generally, the absorption of photons in a molecule is a process of low probability, but if the laser frequency is tuned to a wavelength corresponding to the energy difference between two electronic states of the molecule, the efficiency of the absorption process is increased by several orders of magnitude, which is known as resonance enhancement. The next photon accepted by the intermediate electronic state will now induce the ionization of the molecule, thus rapidly forming radical cations. The time scale for this is determined not by the pulse width of the laser but rather by its intensity. For typical laser intensities of 10^5 W/cm^2, this can take place in less than 100 ps. At a low laser intensity of approximately 10^4–10^5 W/cm^2 or for shorter pulses, the absorption process can be stopped at the level of the molecular ion formation. By increasing the photon density in the focus of the laser beam, absorption of photons in the molecular ion can also be induced and thereby facilitate reactions forming fragment ions. Under these conditions, the system is switching from the absorption ladder of the molecular ion to that of a fragment ion. Further absorption of a few photons in this ion results in further fragmentation producing the next fragment ion. The successive excitation and disintegration of ions can proceed until atomic ions such as carbon are observed.

Although several other mechanisms[48] have been discussed in the literature to explain the fragmentation reactions caused by the multiphoton interaction, for typical systems in multiphoton ionization and fragmentation the *ladder switching mechanism* has been established. This has been confirmed experimentally[49] and can be treated theoretically.[50] As shown in Figure 2a, this mechanism rationalizes soft-ionization and tunable-fragmentation processes for most molecular systems in the gas phase.

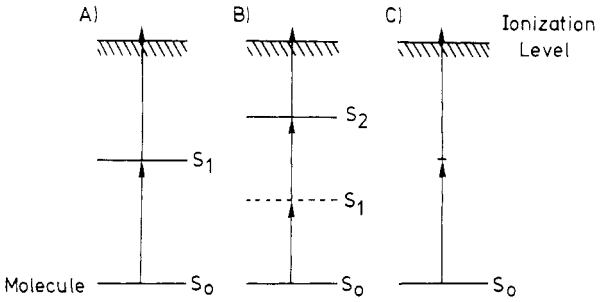

Figure 3. Three possible absorption schemes for multiphoton ionization. Cases A and B show the ionization of a molecule using a real intermediate state. Case C demonstrates the ionization using a virtual intermediate state.

Multiphoton ionization can proceed via different activation schemes to ionize a molecule as shown in Figure 3. So far all examples shown above involve a two-photon resonant activation of the molecule, but even three- or four-photon activation at the same wavelength is possible, as well as combinations of photons with different wavelengths. In cases where the molecule has no chromophoric group, multiphoton ionization can be induced without any intermediate state. It should be noted, however, that this technique requires very high photon densities since a simultaneous absorption of photons in the molecule is rather unlikely. At this point the process becomes indiscriminant, and hence, much important information is lost.

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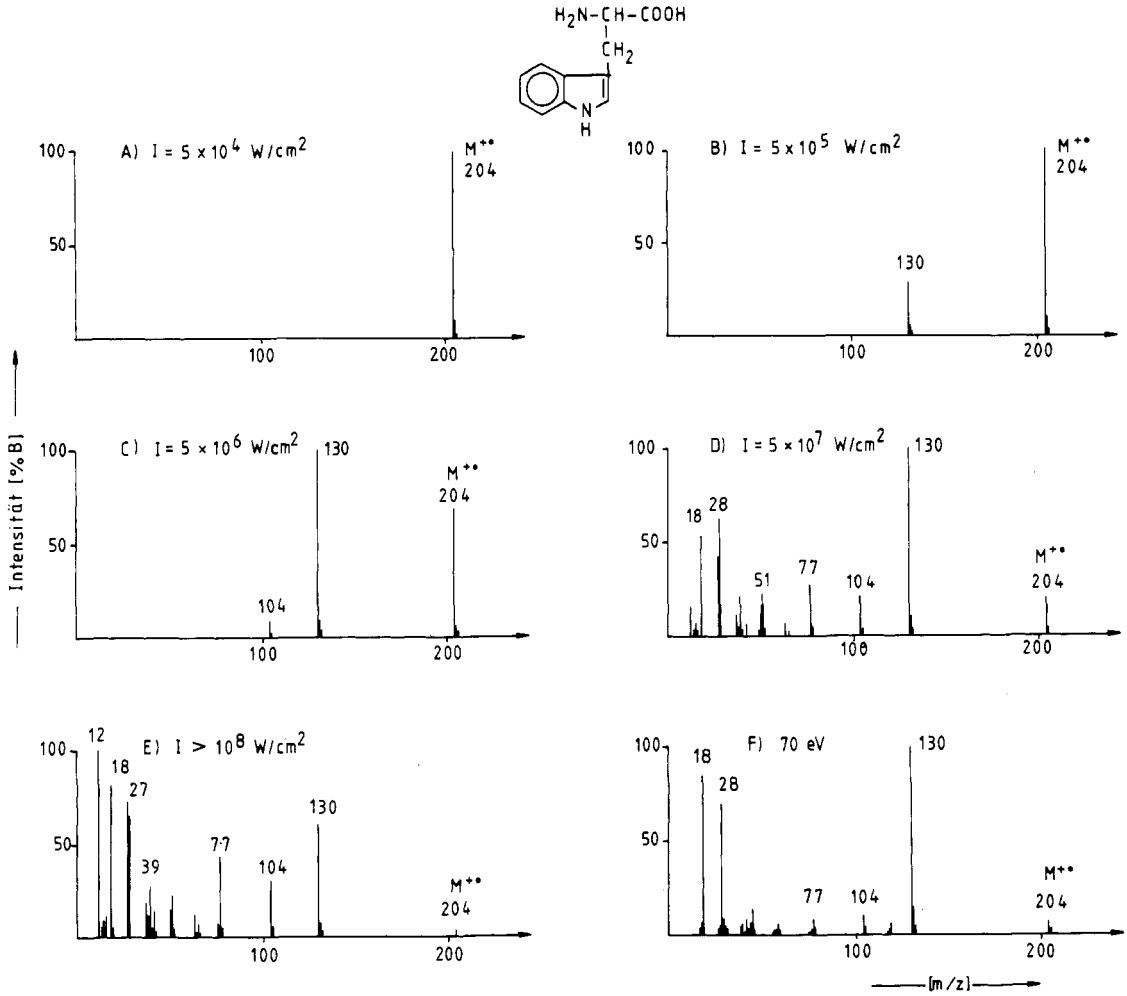

Figure 4. Multiphoton-ionization mass spectra of the amino acid tryptophan. Mass spectra A–E display the effect of increasing photon density in the focus. Mass spectrum F is generated by electron-impact ionization.

The mass separation of the MUPI ions can be carried out in various mass spectrometer systems. The typical systems in the case of MUPI are quadrupole,[51] ion cyclotron resonance,[52] and time-of-flight[53] mass spectrometers. In the experiments described in this Account, a high mass resolution version[54,24] of the reflectron time-of-flight mass spectrometer is used.

Mass Spectra of Biomolecules

With the experimental setup of our laboratory, various kinds of molecules ranging from simple organic compounds, such as aromatic hydrocarbons, to some delicate biological samples, such as peptides, proteins, and others, have been extensively investigated.

Some of the most demanding substances are peptides and proteins which combine within their structures anionic, basic, and neutral residues. Resolving their sequence by mass spectrometric methods constitutes an interesting technique, particularly since it has been shown that these species display specific fragmentation patterns, which permits the deduction of their structure.[59] Traditional experiments often are bound by the reactive sensitivity of the molecular ions, and thus, the resulting products are due to the activation energy and the observation time window. The kind and the intensity of the fragmentations thus can differ widely with the ionization method (energy input) and the detector system (time window) employed. As discussed above, LEIM–MUPI now permits one to adjust the energy to a desired degree of fragmentation and thereby to obtain all the information necessary for an unmistakable identification.

The effect of the increasing photon density can be visualized by the example in Figure 4. Here mass spectra of the amino acid tryptophan at different photon densities are shown and compared to a mass

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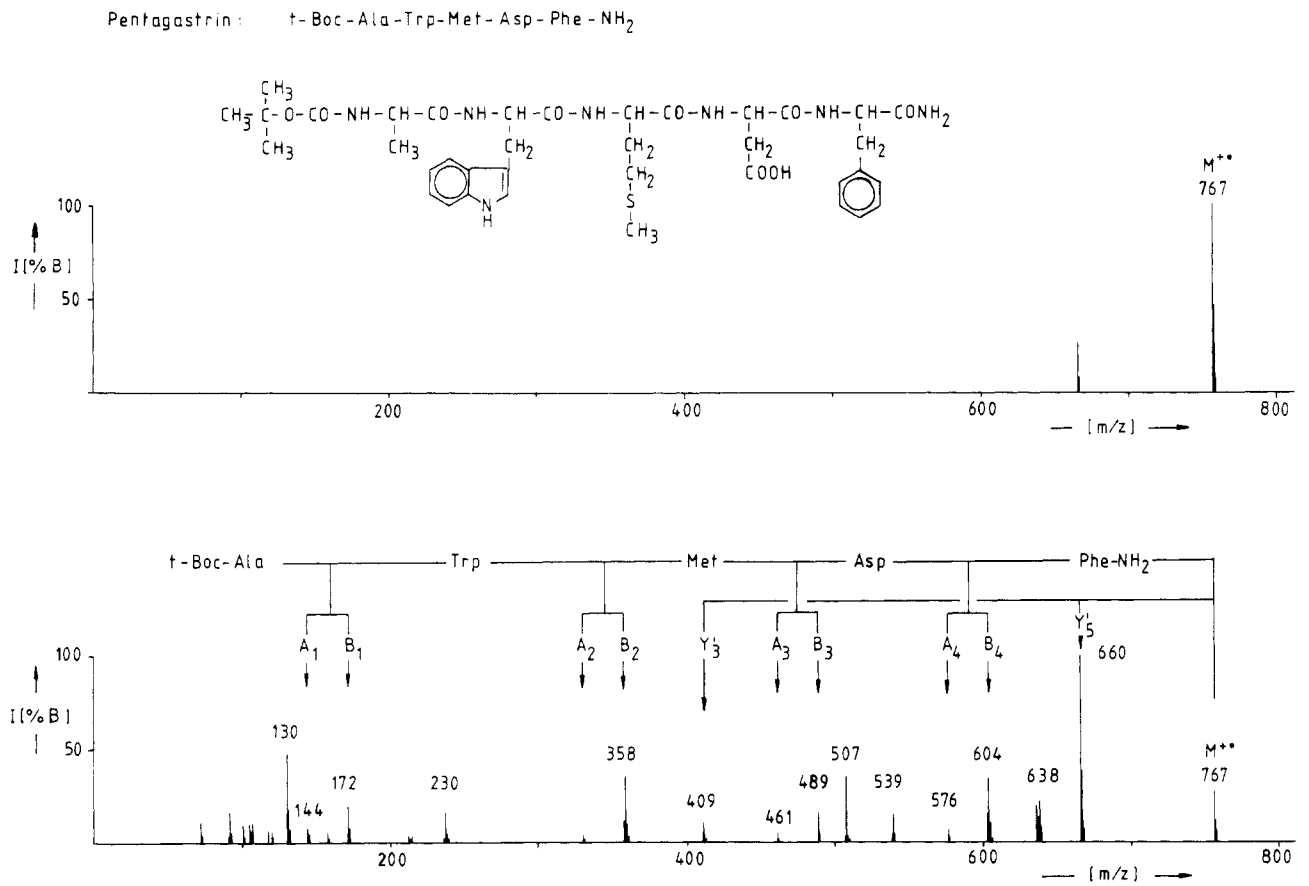


Figure 5. Soft and hard MUPI mass spectra of the pentapeptide pentagastrin.

spectrum of the compound generated by electron-impact ionization. Amino acids, as well as their derivatives, have been intensively investigated by MUPI in the literature.[55] Under electron-impact conditions (Figure 4, F), the molecular ion is formed with very low intensity. The main ions have a mass of 130, the base peak of the mass spectrum. In the lower mass range, ions typical of aromatic compounds are observed at masses 77, 43, 41, and 39.

The different multiphoton-ionization spectra (Figure 4, A–E), taken at an ionization wavelength of 2920 Å, display clearly the influence of the photon density. At low laser powers (Figure 4, A), only the molecular ion of tryptophan at mass 204 was detected. No other ion was observed in the mass spectrum, demonstrating that the absorption of photons in the molecular ion can be avoided at low laser powers. This behavior has also been observed by Levy et al.,[56] but at a different wavelength. However, by increasing the laser power to about 5×10^5 W/cm^2, absorption of at least one photon can occur in the molecular ion, leading to subsequent fragmentation reactions. In the mass spectrum (Figure 4, B), the ion at mass 130 increased noticeably in intensity. This signal stems from the loss of the amino acid function, forming the dehydroindole cation. This reaction is caused by a radical bond cleavage in the β-position to the amino acid function. A further increase of the laser power to approximately 5×10^6 W/cm^2 produces a drop in the intensity of the molecular ion (Figure 4, C) while the signal of the ion at mass 130 is the base peak. In the lower mass range, the first indication of further fragmentations is the observation of reactions in the dehydroindole moiety. When the laser power is increased further to a value of 5×10^7 W/cm^2 (Figure 4, D), the lower mass signals gain dramatically in their intensity. At laser powers of $>10^8$ W/cm^2, the molecule is completely dissociated (Figure 4, E) into fragments as small as the pure atoms. This possibility of completely tunable fragmentation is so far unique to multiphoton ionization and allows an easy and very simple switching between a totally soft and a very hard ionization of the sample.

As examples of such behavior, Figures 5–7 display some soft- and hard-ionization mass spectra of peptides of different lengths and molecular weights ranging from 700 to 5700 daltons (Da). In these cases, the multiphoton ionization occurs through a π–π^* activation in one of the aromatic moieties of either tryptophan, tyrosine, or phenylalanine.[57]

As indicated in Scheme I, MUPI mass spectrometry permits one to sequence peptides in the gas phase due only to the observation of structure-specific ions caused by the breakdown of the peptide bond as well as bonds in the neighborhood of this central bond. These fragments are denoted as to whether they are incorporating the N-terminal end with A, B, and C or, in case of the C-terminal end, X, Y, and Z, as proposed by Roepstorff et al.[58]

We have investigated the fragmentation behavior of peptides in detail with the MUPI method.[59] These investigations result in a clear documentation of soft ionization or tunable fragmentation of these compounds resulting in a clear structural assignment. In Figure 5, the soft and hard mass spectra of the peptide pentagastrin are shown. As in the case of the amino acid tryptophan, the photon density can be tuned to produce only the molecular ion at mass 767. When the photon density is increased, fragmentations are induced. These

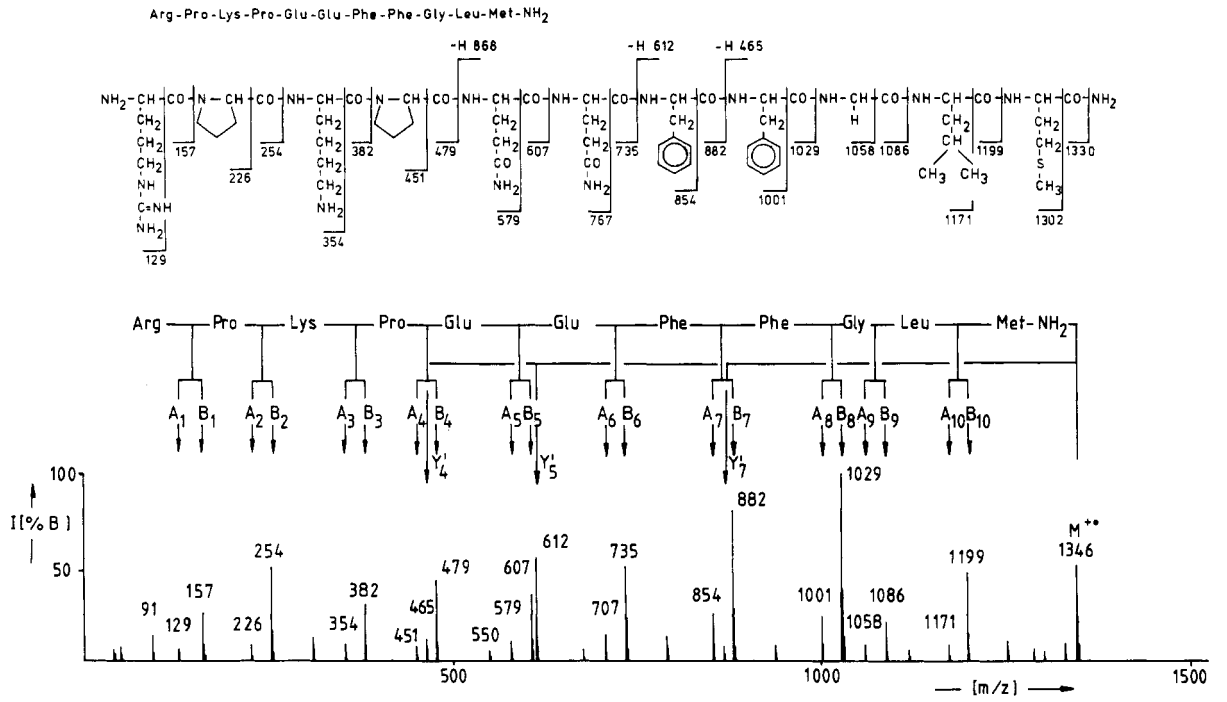


Figure 6. Hard MUPI mass spectrum of substance P.

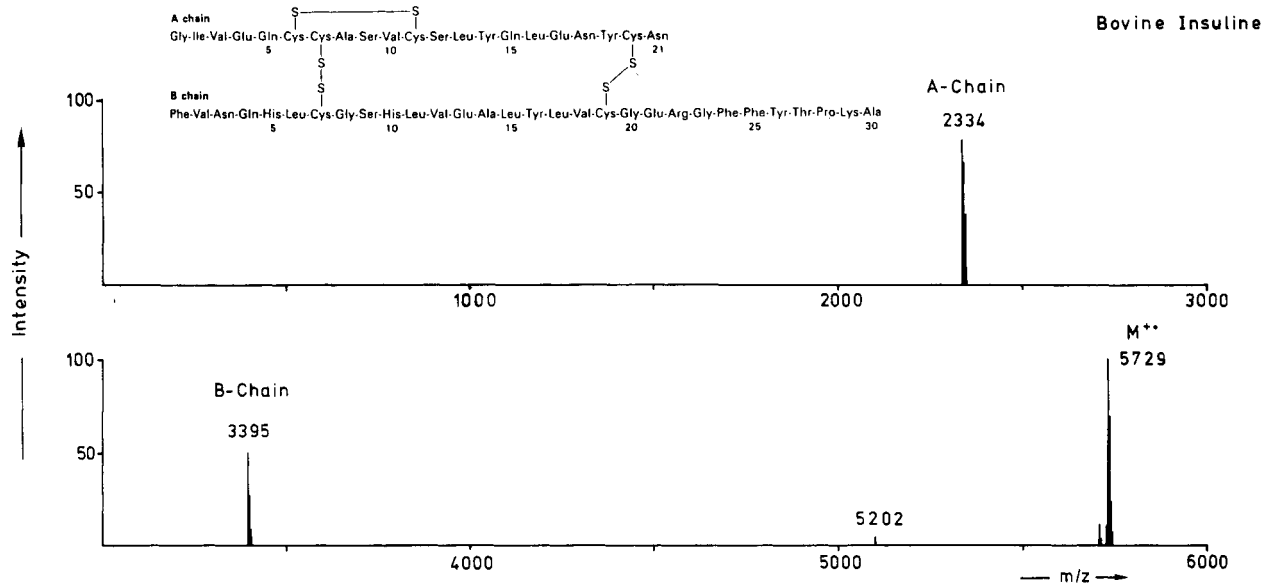
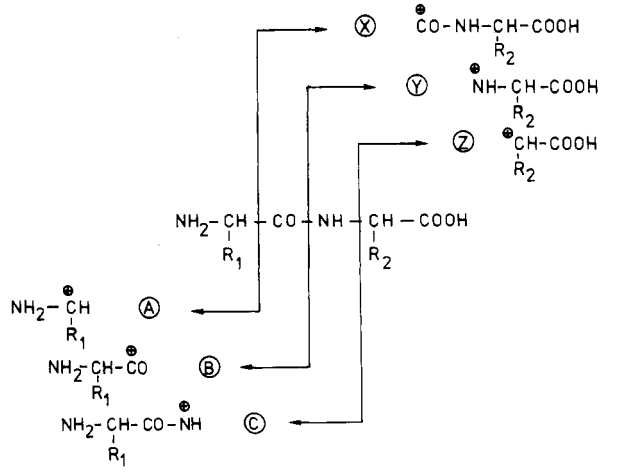


Figure 7. MUPI mass spectrum of bovine insulin.

fragment ions are closely related to the peptide bonds. The A and B type fragments, which are incorporating the N-terminal end of the peptide chain, are found at masses 604, 586, 489, 358, 172, and 101. Besides these sequence ions, some of their counterions also are observed, which are found at masses 666 and 410 (Y′ fragments). As discussed above, these ions include the C-terminal end of the peptide chain.

As a further example of the possibilities in sequencing biopolymers in the gas phase, Figure 6 contains the hard-ionization mass spectrum of the peptide substance P. Here the ionization wavelength is tuned to the $\pi-\pi^*$ transition of the phenylalanine moiety at 2681 Å. The molecular ion of this undecapeptide is found at mass 1346. Again, this mass spectrum consists of a complete set of N-terminal acylium ions of type $(B_n)^+$ and acyl iminium ions $(A_n)^+$, thus resulting in specific mass doublets with 28 mass units difference from which the

Scheme I
Principal Structure-Specific Fragmentation of Peptides



sequence can be deduced unambiguously. Few fragment ions of the $(Y_n')^+$ series are observed at m/z 868, 612, and 465, giving a definite identification.

The base peak of the spectrum at mass 1029 is accompanied by the result of the McLafferty rearrangement close to the aromatic amino acid phenylalanine. The identification of the amino acid in position 10 as leucine rather than isoleucine is based on the appearance of the ion m/z 1129, which represents a loss of 42 mass units from the acyl iminium ion at mass 1171. This McLafferty type rearrangement eliminates a part of the side chain as propene, while isoleucine would eliminate ethylene. The mass differences of 128 mass units between ions $(B_6)^+$ and $(B_5)^+$ and between $(B_5)^+$ and $(B_4)^+$ can be interpreted as glutamine and not as lysine. This is based on the fact that $(B_{n-57})^+$ ions, found at masses 678 and 550, are favored by the former. The $(B_3)^+$ ion containing the lysine residue shows no such fragment ion, but does produce one at mass 310. This ion corresponds to an elimination of the intact lysine side chain from the $(B_3)^+$ ion.

The mass spectrum of bovine insulin, shown in Figure 7, is obtained with a laser output power of approximately 10^7 W/cm^2.[57] The excellent signal-to-noise ratio, which is generally observed in multiphoton-ionization mass spectra, allows detection of even fairly low intensity signals like the loss of a methyl group (m/z 5912). The molecular ion of the compound is detected at mass 5929.45 (calcd 5729.67 Da). Two additional intense signals are identified as the A chain at 2334.39 Da and the B chain at 3895.41 Da. These fragment ions are due to the breakdown of the two disulfide bridges in the insulin molecular ion. The softness of the ionization procedure can again be seen by the fact that the molecular ion is the base peak of the mass spectrum.

Concluding Remarks

This Account has described only some of the many capabilities and results currently available by combining laser evaporation of intact neutral molecules with multiphoton ionization and mass spectrometry. The MUPI mass spectra of many compounds are seen to differ in some profound and particular characteristics ways from mass spectra obtained by other ionization methods. As outlined above, the separation of the desorption and ionization into two spatially and temporally separated processes permits a simple application of this mass spectrometric method to any delicate sample. The advantage of steering the degree of fragmentation in a way unique to this mass spectrometric method suggests that LEIM, together with MUPI, may become an interesting tool for sequence and structure analysis. For analytical purposes, this allows, on a day to day analytical basis, the quick and facile identification of the molecular weight of a sample together with direct information on its structure. The simultaneous availability of extremely high sensitivity could make this multiphoton mass spectrometry a versatile tool for trace and pollutant analysis.

The production of neutral molecules with interesting structures and large molecular weight in the gas phase and in a molecular beam can open new opportunities not only for mass spectrometry but also for all other investigation methods requiring neutral, vaporized molecules, such as absorption spectroscopy. The application of this method to questions of molecular structure, reactivity, and photochemistry has just begun.

We thank the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the Bundesministerium für Forschung und Technologie for their continuing financial support of this work. A large group of scientists have worked over years to make multiphoton ionization a versatile method for mass spectrometry, among them H. J. Neusser U. Boesl, K. Walter, R. Weinkauf, J. Lindner, C. Köster, and R. C. Beavis. We indebted to their continuing help, interest, and advice. We thank Prof. G. H. Attkinson, on leave from the University of Arizona, for his critical reading of the manuscript.

Registry No. L-Tryptophan, 73-22-3; pentagastrin, 5534-95-2; substance P, 33507-63-0; insulin, 9004-10-8.