Journal of Natural Products
Vol. 47, No. 2, pp. 215-225, Mar-Apr 1984

FAST ATOM BOMBARDMENT AND MIDDLE MOLECULE MASS SPECTROMETRY[1]

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ABSTRACT.—A number of recent instrumental advances have made possible the extension of mass spectrometric analysis to molecules in the middle mass region, 1000 to 10,000 Daltons. Among these, fast atom bombardment has proven to be the most effective ionization technique. It is also an important technique for analysis of ionic and thermally labile compounds. Some features of fast atom bombardment are reviewed, along with examples of applications in structure elucidation, fingerprinting, stable isotope analysis, and quantitation. Some problems associated with reading molecular weight information out of the spectra of heavier compounds will also be discussed.

In the early 1960s, a tremendous boost was provided to studies of natural products by the development of the direct insertion probe as an alternative to the gas expansion chamber for introducing samples into a mass spectrometer. This technologic advance launched work with steroids and alkaloids in mass spectrometry laboratories around the world and also generated a lot of new sales for the instrument manufacturers. The direct probe permitted less volatile samples of molecular weights above 300 to be vaporized, ionized, and analyzed (1).

The extension of mass spectrometry to analysis of heavier samples has continued to be a developmental goal. This article reviews an ionization technique that has again been readily adapted by laboratories all over the world and that provides ionization of compounds with molecular weights in excess of 3000. Some of the novel aspects encountered in spectra of compounds weighing more than 3000 will be mentioned. Important capabilities of fast atom bombardment (FAB) will also be pointed out for classes of compounds other than high mass samples.

There are four areas in which mass spectrometry can provide answers to questions: isotope analysis, quantitation, fingerprinting, and structure elucidation (2). All four kinds of applications are found in the natural products literature, the first two especially in studies of biosynthetic routes. In structure elucidation, mass spectrometry is usually used in conjunction with other chemical and instrumental techniques, the most important single piece of information provided by the mass spectrometer being the molecular weight.

There are at least four considerations in developing instrument systems to permit assessment of molecular weights above 3000 amu (3). These heavier samples must be ionized, the ions must be separated/analyzed, the ions must be detected, and the spectra must be counted and interpreted. Striking advances have been made in recent years in all four areas, including the development of computer support for averaging multiply acquired spectra and assigning masses through an extended mass range. Active development continues all around the world.

THE FAST ATOM BOMBARDMENT TECHNIQUE.—In the FAB technique (4,5), the sample is presented in a liquid matrix of low volatility and desorbed by "bombardment" with a high flux of neutral particles such as argon or xenon atoms. Examples of the effec-

[1]Presented as a plenary lecture at the "Recent Developments in NMR and Mass Spectral Analyses of Natural Substances," Symposium of the 24th Annual Meeting of the American Society of Pharmacognosy at the University of Mississippi, Oxford, Mississippi, July 24-28, 1983.

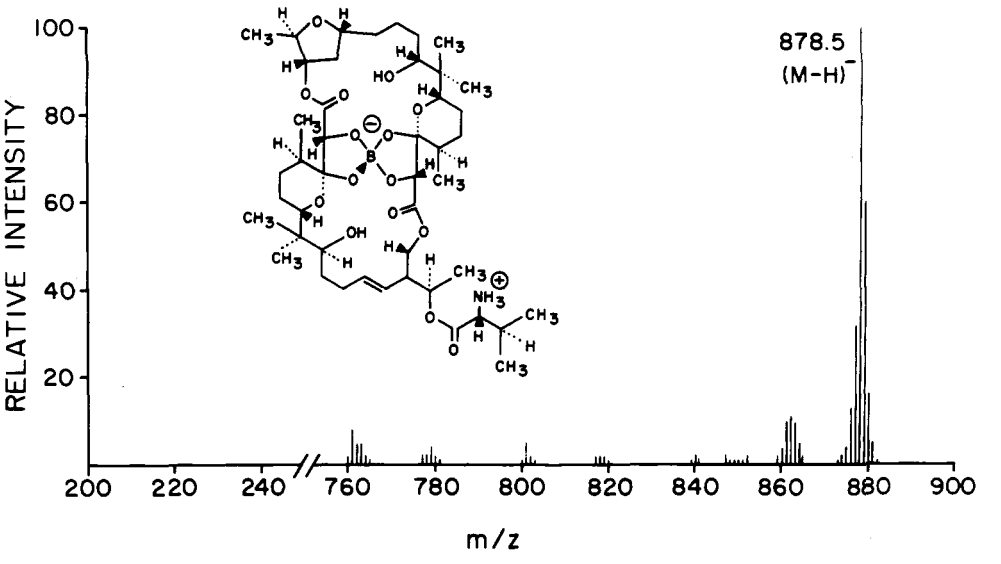


FIGURE 1. Negative Ion FAB mass spectrum of boromycin (sample courtesy of Heinz Floss).

tiveness of this approach are shown in Figures 1-3. The technique has provided successful analyses for many heretofore refractory organometallics. Working with a sample provided by Heinz Floss, we found mass spectra could be obtained by boromycin (6) and related compounds by FAB (Figure 1) and also by field desorption. An example where field desorption failed but FAB provided a facile analysis is shown in Figure 2,

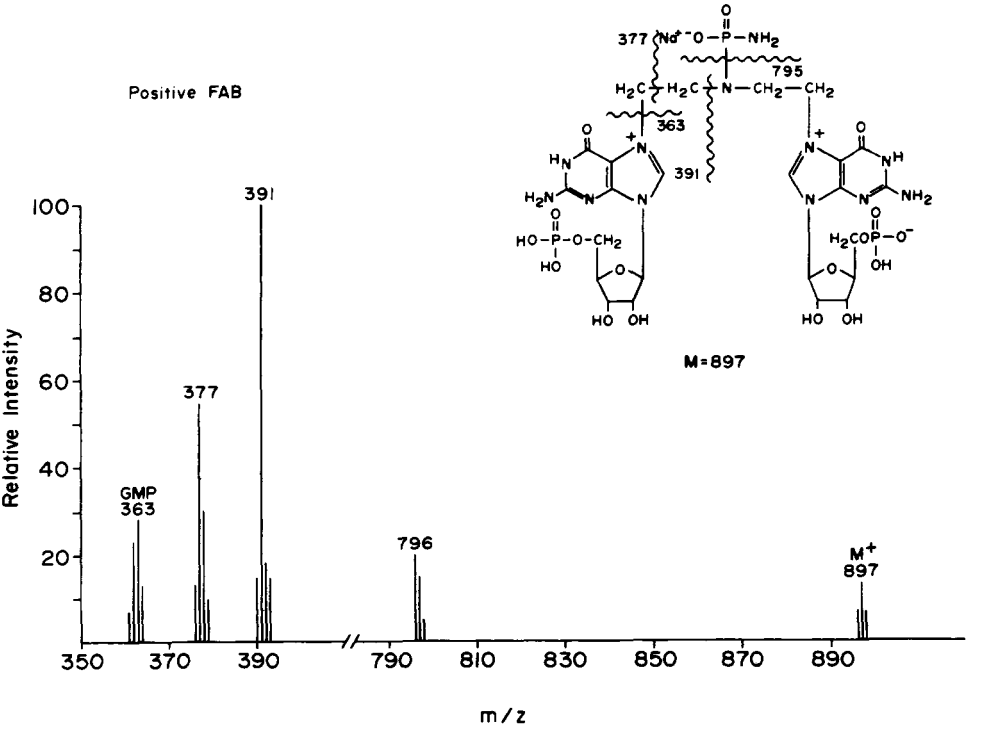


FIGURE 2. FAB mass spectrum of guanosine monophosphate crosslinked by phosphoramide mustard (7) (by permission of the American Chemical Society).

the spectrum of guanosine monophosphate cross-linked by a cytotoxic mustard generated in antitumor drug therapy (7). In addition to molecular weight information, structural features can be deduced from fragment ions in this spectrum. Figure 3 contains a part of the FAB spectrum of a diphosphorylated fraction of the Lipid A Lipopolysaccharide complex purified by Kuni Takayama from a mutant of *Salmonella typhimurium* (8).

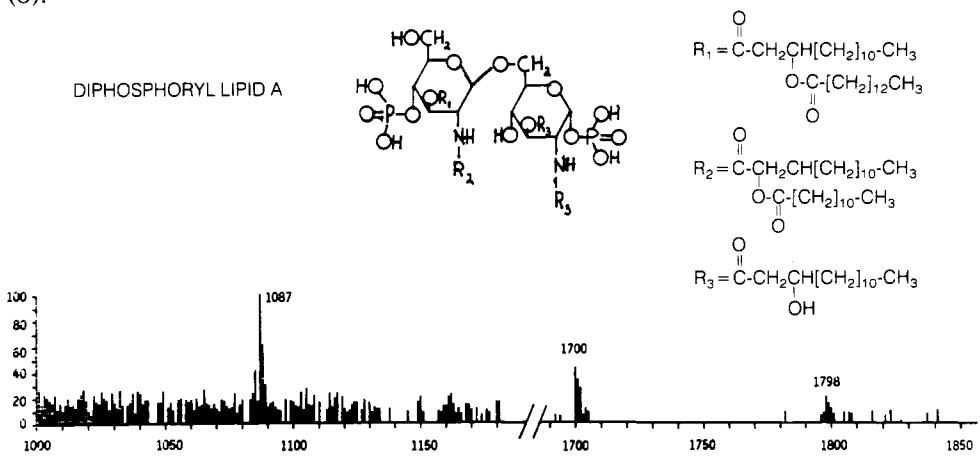


FIGURE 3. FAB mass spectrum of a diphosphorylated fraction of the lipid A complex (8).

It can be seen from this rapid survey that this technique is effectively used with samples which are ionic, involatile, thermally unstable, and/or relatively heavy. This set of sample characteristics makes it highly complementary to electron impact. It should be noted also that, although there is a large area of overlap in samples susceptible to field desorption and to FAB there are situations in which field desorption has been found preferable (9) and *vice versa*.

The source geometry and the role of the fluid matrix in FAB have been discussed (*e.g.*, 10-13). The source schematic in Figure 4 (14) indicates that positive ions, nega-

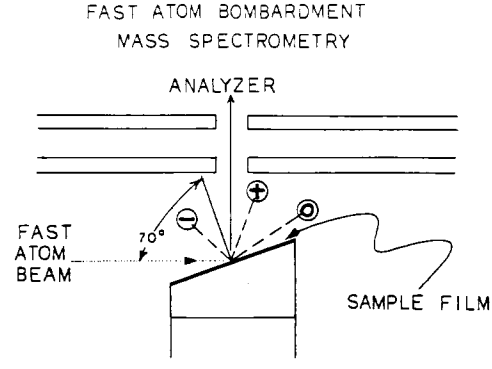


FIGURE 4. Schematic of a fast atom bombardment source (14) (by permission of Elsevier).

tive ions, and also neutral species are desorbed by the bombardment. Figure 5 illustrates schematically the several roles of the nonvolatile fluid matrix. Sample molecules can be ionized by chemical reactions in solution. Solvation provides charge separation, lowering the energy required for desorption. Solution equilibria lead to replacement of active protons with ubiquitous alkali metal cations. Solvent and sample molecules directly impacted by bombarding atoms suffer damage reminiscent of radiolysis (15); however, sufficient energy is transferred by the impact through the matrix (by processes

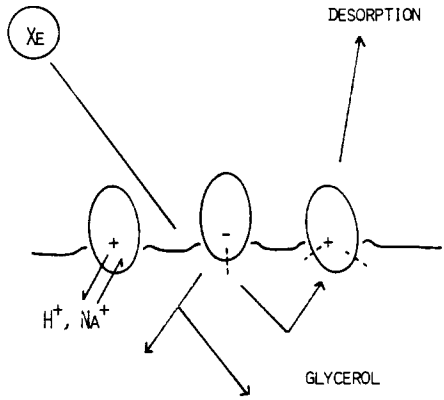


FIGURE 5. Schematic representation of the fluid matrix (14) (by permission of Elsevier).

as yet generally described only for hard crystals) to desorb preformed ions, molecules, and clusters from the surface. A final contribution of the fluid matrix is constantly to bring new sample ions to the surface so that ion desorption can be even and prolonged.

The experimental variables available to the chemist in a FAB experiment on a commercial instrument include selection of the particles and energy of the primary (bombarding) beam and selection of the fluid matrix and its supporting stage. It should be noted that the bombarding atoms can also be ions with no change in the physics of the energy transfer, however with some implications for engineering the instrument (16). The happy conclusion that the ions detected in the spectrum are related to the ions present in solution allows us to control and optimize the spectrum by manipulating the chemistry of the solution. Molecular ion species are usually even electron ions such as $(M-H)^-$, $(M+H)^+$, or $(M+Na)^+$. The addition of acid or use of more acidic thioglycerol, instead of the usual glycerol, as the matrix increases the sensitivity of the technique for peptide cations. Use of ammonium salts or a basic matrix has been suggested to enhance detection of anions formed from phosphate, sulfate, or carboxylate groups. Addition of salts, such as LiCl, to the matrix is sometimes found to enhance production of $(M+Li)^+$ ions, especially with compounds that are not readily ionized.

Arguments have been made based on lattice energies (17) and on actual experiments with thermal laser and field desorption (18) that less energy is required to desorb quaternary ammonium ions (as examples of preformed organic cations) than $(M+Na)^+$, than $(M+H)^+$, than M^+. It is not surprising, then, to find that different compounds have different detection limits or desorption efficiencies in FAB. In one comparison in our laboratory, we found that the molar amounts of a tetrapeptide, a phospholopid, and a tetranucleotide required to produce M+H ions with a signal to noise ratio exceeding 5 varied through three orders of magnitude.

Although practitioners of secondary ion mass spectrometry have used neutral particles (atoms) in their incident beam for at least 20 years (19,20), the introduction of the fluid matrix, which is the distinguishing and novel feature of the so-called FAB technique, permits the use of a high flux primary beam and generation of a strong, steady, and prolonged secondary (sample) ion current. Sample ion currents have been found to be comparable to those generated by electron impact. As a consequence, all the ancillary techniques of the mass spectroscopist are readily compatible with FAB: computerized acquisition of data (21,22), high resolution, linked scan recording of metastable spectra, and use of the tandem ms/ms techniques.

INFORMATION CONTENT OF THE SPECTRA.—Fast atom bombardment spectra

have been used to provide molecular weights, to provide structural information through fragmentation, for stable isotope analysis (including quantitation with isotope-labeled internal standards) and for quantitative and qualitative analysis of mixtures.

The positive-ion and negative-ion spectra of guanosine monophosphate alkylated by a therapeutic nitrogen mustard may be contrasted in Figure 6. The replacement of acidic protons by various alkali earth cations results in the presence of several molecular ion species in the positive ion spectrum. Because the anions detected in the negative ion spectrum are formed by loss of these protons or counter ions, the molecular ion region is simplified. Salts can be removed from the sample on an ion exchange column to simplify the positive ion spectrum (23). The presence of several molecular ion species at appropriate mass intervals does provide confirmatory readings on the molecular weight. It also reduces intensities relative to a single signal. One can promote controlled replacement in order to count active protons. This appears to work well for up to four protons (23). Because alkali metal cations are ubiquitous contaminants, they may interfere with fingerprint comparisons of samples from two different sources. For example, measured spectra of synthetic leukotriene C_4 and of leukotriene C_4 isolated from murine mastocytoma cells (24) each contained MH^+, $(M+Na)^+$, and $(M+K)^+$ ions; however, they had different relative abundances.

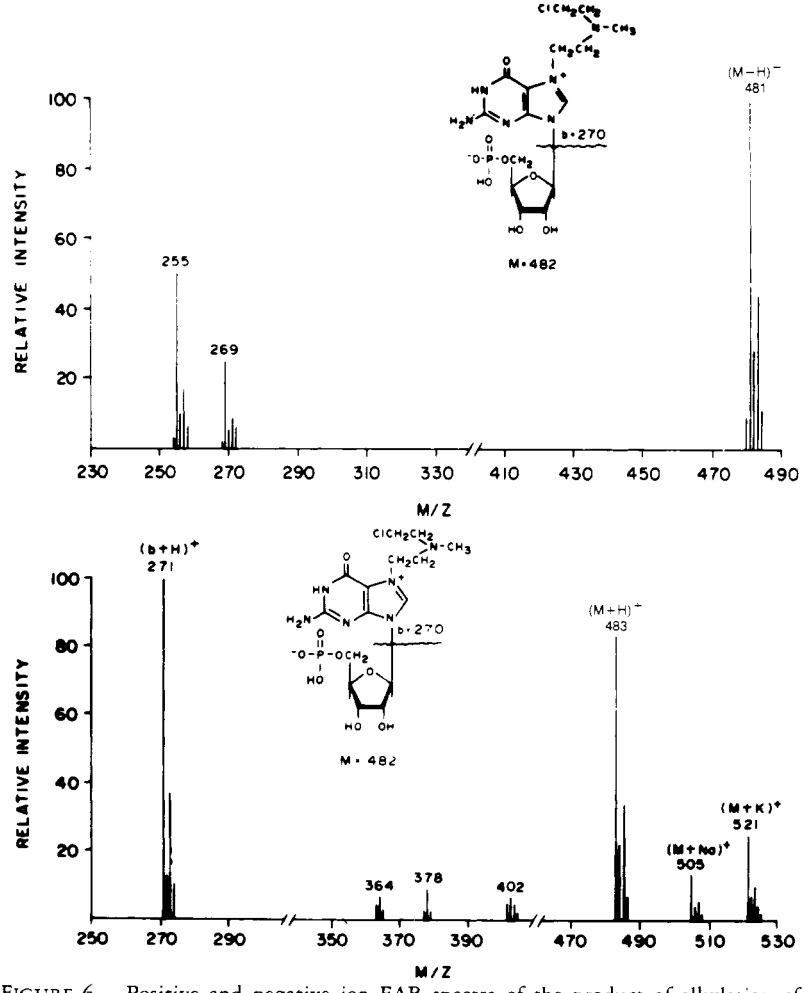


FIGURE 6. Positive and negative ion FAB spectra of the product of alkylation of guanosine monophosphate by methyl bis(2-chlorethyl)amine (24).

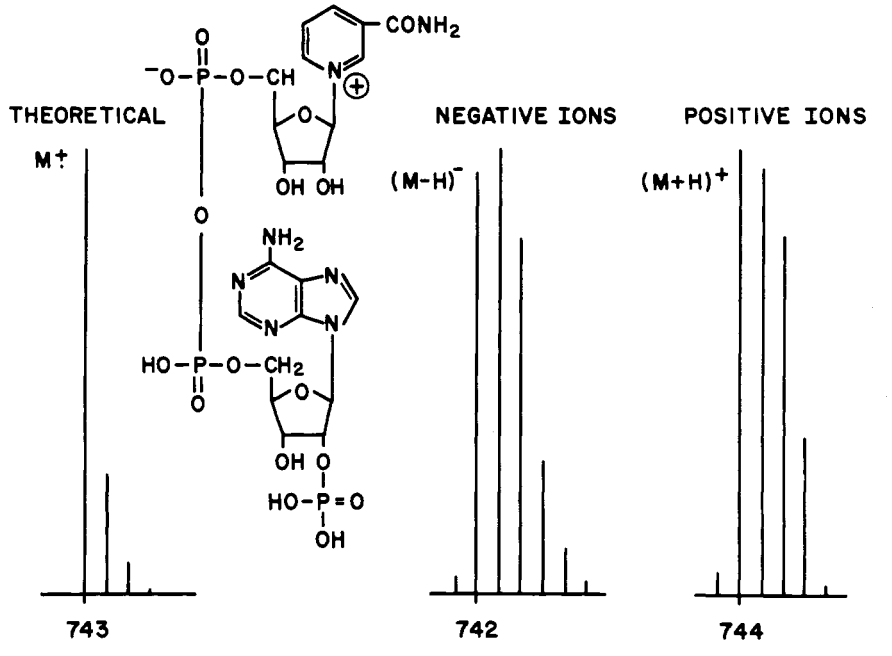


FIGURE 7. Partial FAB spectra of a sample of oxidized nicotine adenine dinucleotide phosphate.

Less frequently, reduction of samples has been observed in the liquid matrix under high flux bombardment. Figure 7 contains the positive and negative molecular ion regions of NADP analyzed in glycerol, certified >95% oxidized by an enzymatic assay. Extensive one- and two-electron reduction is visible.

Fragmentation does occur under FAB, and, although processes resembling hydrolysis, solvolysis, and pyrolysis are prominent, decompositions observed as metastable ions in the spectra confirm gas phase unimolecular decomposition as well. Sequence fragmentation has been reported for peptides, nucleotides, and polysaccharides. In the peptide area, where experience has been accumulated in many laboratories, it has become clear that not all sequence ions are always present, perhaps because not all amide linkage are cleaved equally readily (21,25,26).

An interlaboratory comparison of spectra run on a variety of instruments with a uniform matrix recipe, and using aliquots of the same sample, suggests that FAB spectra are no less reproducible than electron impact spectra (27).

Figure 8 presents the negative ion spectra of a glycosidic cofactor, uridine diphosphoglucuronic acid, and of analogs partially labeled with ^{18}O. Analysis of the molecular ion regions provides facile assessment of the isotope population, and analysis of the various fragment ions—notably those of mass 403—leads to the conclusion that isotopioc oxygen has been incorporated into the sugar acid moiety exclusively (28). Glucuronic acid was transferred under enzyme catalysis from the cofactor to a substrate, *p*-nitrophenol, and the isotope population of that new glycoside was assessed by mass spectrometry. The FAB analysis may be compared in Table 1 with analyses made using field desorption and electron impact (derivatized sample). Agreement is satisfactory, within the usual uncertainty of scanning measurements on heavy molecules (2). Thus, FAB appears to have potential for analysis of isotope labels in studies of the biosynthesis of polar compounds.

In another application of stable isotopes, FAB has been used to quantitate surfac-

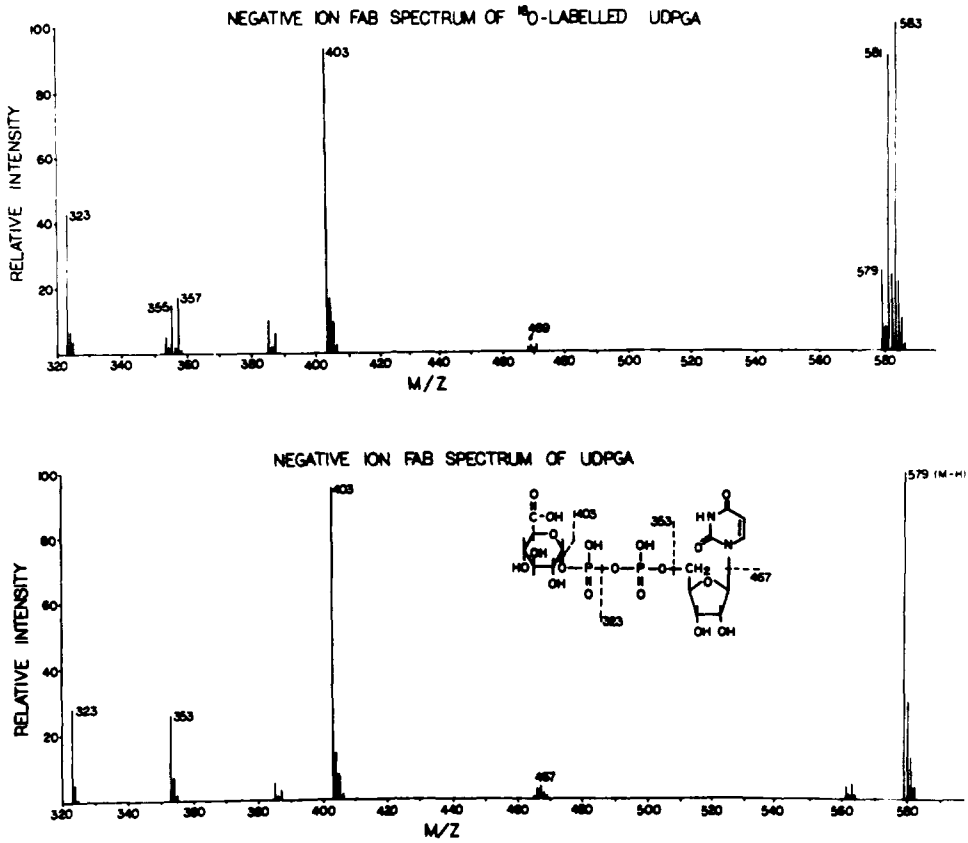


FIGURE 8. FAB spectra of uridine diphosphoglucuronic acid and of ^{18}O-labelled analogs (28).

tant dipalmitoyl phosphatidylcholine extracted for amniotic fluid to which non-adeuterio-dipalmitoylphosphatidylcholine had been added as internal standard (29).

The potential of FAB for qualitative and quantitative analysis of mixtures has been debated from its inception. The large differences discussed above in desorption efficiencies (sensitivities) of compounds with different functional groups appear to limit appli-

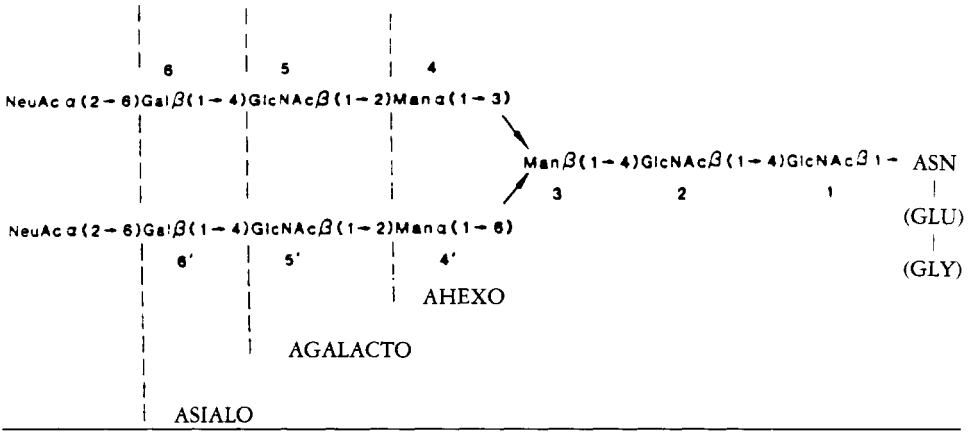
TABLE 1. Isotope Analysis by FAB, Field Desorption and Electron Impact[a]

p-Nitrophenol Glucuronide	Fast atom Bombardment (%)	Field Desorption (%)	Electron Impact (%)
Unlabeled	60	59	58
Mono-isotopic labeled	34	35	34
Di-isotopic labeled	6	6	8

[a]All values are calculated with an estimated uncertainty less than or equal to 3%, *e.g.*, 60±3.

cations only to mixtures experimentally well-defined. Recently, we have evaluated protocols for quantitative assessment of glycopeptide mixtures from human fibrinogen (30). These species have heterogeneity in both the amino acid moiety and the polysaccharide moiety (see structure). Table 2 compares the amino acid analysis from a total hydrolysate with the amino acid analyses derived from abundances of ions generated by FAB on the intact glycopeptide mixture (HFG) and on mixtures resulting from removal of all muraminic acid units (asialo), from cleavage of the galactosyl units (agalacto), and

TABLE 2. Amino Acid Composition[a] of Glycopeptides Resulting from Sequential Exoglycosidase Treatment



	FAB-MS				HYDROLYSIS
	HFG	ASIALO	AGALACTO	AHEXO	HFG
ASN	1.00	1.00	1.00	1.00	1.0
GLU	0.44	0.45	0.39	0.38	0.4
GLY	0.09	0.06	0.05	0.05	0.2

[a]Normalized to ASN content.

from cleavage of *N*-acetylglucosamine units in the biantennary chains. These analyses are reasonably consistent, allowing for commonly encountered artifactual contamination by glycine in the amino acid hydrolysis and suggest that, for this particular mixture, relative peak heights reflect the relative composition. This was not the case for carbohydrate heterogeneity, and standard curves had to be constructed by mixing known amounts of purified asialo, monosialo, and disialo polymers (31).

CONSIDERATIONS FOR HEAVIER SAMPLES.—Fast atom bombardment has allowed mass spectroscopists to work much more easily in the mass range opened earlier by electron impact and field desorption (3), up to about 4000 amu. It has also allowed us to move rapidly towards 10,000 (22,32) amu and has brought the limitations of currently available analyzers and detectors into sharp focus. While the instrumentalists incorporate post accelerators into their detection systems and evaluate new principles for magnet-based and time-of-flight analyzers, the chemist might well begin to evaluate the spectra we are beginning to see of compounds with molecular weights exceeding 5000 amu. Recently, we have addressed the question: how should molecular weights be calculated and reported from these spectra?

In Figure 9, the molecular ions are displayed and calculated (33) for glucagon and a glucagon trimer. Both of these spectra contain a number of molecular ion species, reflecting primarily the natural abundance of ^{13}C, but also of ^{2}H, *etc.* The most abundant ion in the molecular ion envelope of glucagon is that which contains two atoms of ^{13}C. In the hypothetical trimer, the envelope has become nearly symmetrical, and the three most abundant species are present in nearly equal amounts. It can be seen that the nominal mass, calculated from the masses of the most abundant isotopes of each constituent element rounded off to the nearest whole mass (*e.g.*, C=12, H=1, falls below the

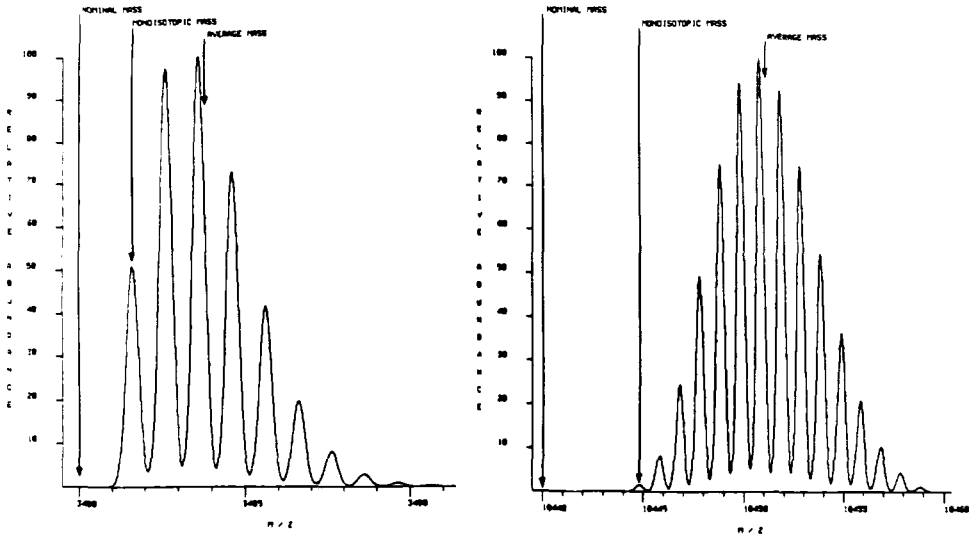


FIGURE 9. Molecular ions calculated for glucagon, $C_{153}H_{224}N_{42}O_{50}S$ and a trimer of glucagon, $C_{459}H_{672}N_{126}O_{150}S_3$ (32).

actual masses of any of the molecular ions. The monoisotopic mass, calculated from the exact masses of the most abundant isotopes of each constituent element and reflecting the mass defect (*e.g.*, C=12.000, H=1.073), is readily discernible, though not the most abundant ion in the group of molecular ions of glucagon. Its relative abundance is less than 5% for the trimeric peptide of mass 10,444, and would be distinguished with difficulty above background in a FAB spectrum. After evaluating many molecular ion envelopes in this mass range, both theoretically calculated and also experimentally determined, we have suggested (22,34) that the most meaningful characterization of the molecular weight of heavier compounds will be the average molecular weight. It should also be pointed out that each peak in these envelopes may represent a number of isobaric contributions. At least 13 different formulae contribute ions to the most abundant peak in the bovine insulin molecular ion envelope (Figure 10), ranging from *m/z* 5733.602 for the formula containing one ^{15}N and one ^{34}S to 5733.621 for that containing one ^{13}C and one ^{2}H.

If the average molecular weight is the value most meaningfully measured and reported from the spectrum of a compound above 5000, the next question naturally arises: what resolution is required to get a good value? Do we need to give up sensitivity in some analyzers to maintain unit resolution? Figure 10 shows the molecular ion envelopes of porcine insulin determined using FAB at unit resolution ($\sim$5800) and at low resolution ($\sim$1500). Both spectra are acquired by computer, and a number of scans are averaged before peaks are centroided or masses assigned. Ion counts shown on the vertical axis provide estimates of sensitivity that may be compared. Average molecular weights were determined from each envelope with computer support. Table 3 compares the accuracy and precision of average molecular weights based on eight samples separately inserted and measured at unit resolution and on five separate determinations at low resolution. Accuracy is comparable. Precision is better at low resolution, the method with the greater secondary ion current (sensitivity). Comparisons of this sort have been made for a larger set of compounds and confirm the suggestion (22) that unit resolution may not always be required to provide the best assessment of molecular weights of unknown samples in this middle mass range.

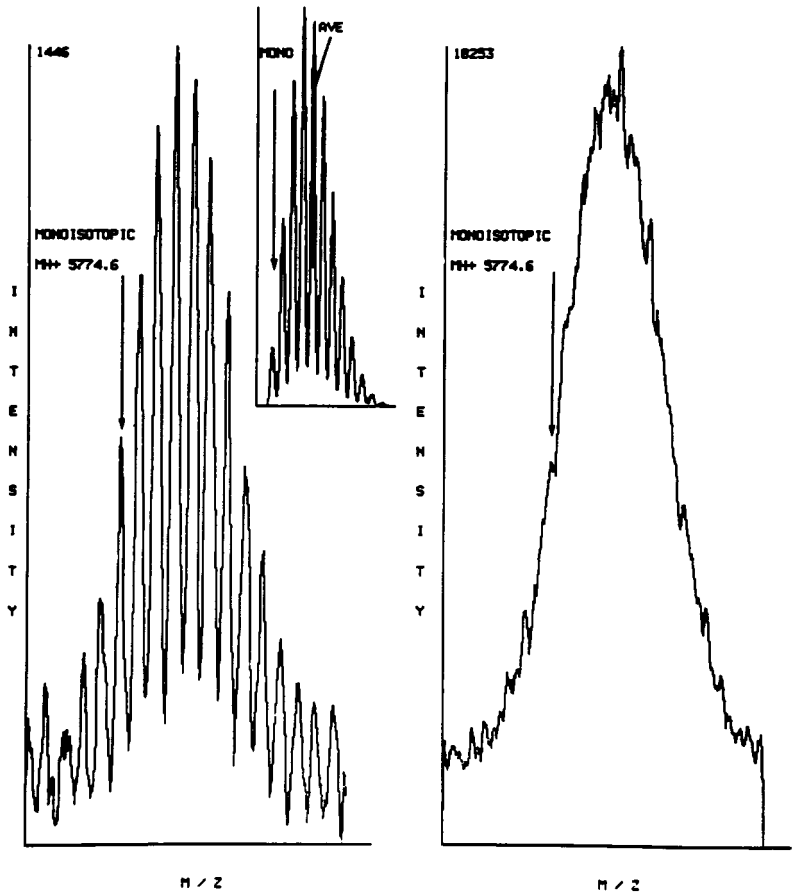


FIGURE 10. Partial FAB spectra of porcine insulin measured at unit resolution and at low resolution. The insert shows the theoretical molecular ion envelope (22).

TABLE 3. Average Mass of Porcine Insulin

EXPERIMENTAL		THEORETICAL
Unit Resolution	Low Resolution	
5779.00 +.41 s=.53 (8)	5778.96 +.37 s=.22 (5)	5778.59

ACKNOWLEDGMENTS

Many colleagues have contributed to the work at Johns Hopkins reported here. I especially want to thank Professor R.J. Cotter, Dr. J.A. Yergey, and Mr. D. Heller, as well as the National Science Foundation for support of the Middle Atlantic Mass Spectrometry Facility.

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