Electrospray ionization–principles and practice

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INTRODUCTION

Chemistry has its origins as a quantitative science in the careful weighing of products and reactants by Lavoisier and his followers beginning some 200 years ago. Ever since then, the constantly evolving gravimetric balance has been a faithful servant of the laboratory chemist and has played a major role in developing the analytical methods that are the foundation of contemporary chemical science. Perhaps the ultimate stage in the evolution of that balance is represented by the modern mass spectrometer. It is able to determine with high precision the masses of individual atoms and molecules by transforming them into ions and measuring the response of their trajectories in vacuo to various combinations of electric and magnetic fields.

Clearly, the *sine qua non* of such mass determination is the transformation of analyte atoms and molecules from their initial state in a sample to ions in vacuo ready for "weighing." Over the years, ingenious investigators have produced a variety of methods for achieving this transformation. One of them, *electrospray (ES)* ionization, has recently shown itself capable of producing intact ions, with multiple charges, from remarkably large, complex, and fragile parent species. Our assignment here is to review what has thus far been learned about this still uncommon technique and what it seems able to offer practitioners of mass spectrometric analysis. Our approach will be to set forth the present state of the ES ionization art in terms of a sort of menu of its procedures, processes, performance, and promise. Until very recently we have been almost the only group that has worked with ES ionization since the pioneering efforts of Malcolm Dole (1) some 20 years ago. Consequently, this review is more tutorial than most. Moreover, it may seem like a cook book that is overly preoccupied with the authors' own culinary adventures. The reason is that many of the dishes to be described were first tried out in our own kitchen. Therefore, we earnestly urge the reader to remember what every gourmet knows: the piquancy of any dish on a bill of fare is due much less to its ingredients than to the skill of the chef who prepares it.

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Thus, we are confident that other "cooks" will have even more success than we have had with the recipes to be presented.

The first course on the menu constitutes a brief survey of methods that have been used to produce ions from neutral parent species. Next we serve up a description of the essential features of the apparatus and procedures underlying ES ionization. There follows a digest of selected results. Finally, in an epilogue, we set forth a number of recent developments that have emerged since this article was written and submitted for review.

II. SURVEY OF IONIZATION METHODS

Our meal begins with an appetizer comprising that alphabet soup of techniques that mass spectrometrists have found useful in transforming neutral species into charged particles whose mass relates in a known way to those of the parent molecule.

A. Ionization of volatile species

To produce ions in vacuo from species that are naturally gaseous, or that can be vaporized without decomposition, is relatively straightforward. One simply disperses them as a gas or vapor and brings about sufficiently energetic encounters of their component molecules with electrons as in electron ionization (EI), with photons as in photo ionization (PhI), with ions of other species as in chemical ionization (CI), or with electronically excited neutral species as in Penning ionization (PeI). These ionizing encounters usually result in the loss of an electron by the analyte species to form positive ions, but sometimes ionization results from attachment of species with a positive or negative charge. The encounters are most often brought about in a region having a gas density sufficiently low to insure that ions entering the mass analyzer's vacuum chamber will not be accompanied by enough neutral molecules to raise the latter's background gas density above tolerable levels. However, with CI or AI it is sometimes advantageous, and with ES essential, to carry out the ionization step at substantially higher gas densities. The fraction of ions produced that can be delivered to the analyzer then depends upon how much accompanying neutral gas can be accommodated by the vacuum chamber's pumping system. Even when this fraction is very small, as is the case for ionization at atmospheric pressure, overall signal/noise may be higher than can be achieved when ionization occurs at lower densities and a larger fraction of the ions produced can be introduced to the mass analyzer.

B. Ionization of non-volatile species

For the far larger class of species that cannot generally be vaporized without substantial, even catastrophic decomposition, the problem of producing intact ions is much more refractory. The usual procedures that have been developed for EI, CI, PhI, and PeI are not directly applicable because they require a gas-phase dispersion of relatively stable molecules. Thus challenged, intrepid and

ingenious investigators have responded with a number of "soft" ionization techniques that have been remarkably effective in extending the power of mass spectrometric methods to the analysis of large, complex and fragile molecules that play such an important role in many areas of science and technology, especially biology and medicine. These techniques fall into two general categories to be discussed in what follows.

1. Energy sudden methods

The techniques in this group derive from an idea originally proposed by Beuhler et al. (2). On the grounds of rate theory for unimolecular decomposition they argued that sufficiently rapid heating could vaporize complex molecules before decomposition occurred. In some demonstration experiments they showed that indeed fragmentation decreased as heating rates increased.

Then, in developing so-called "pyrolysis mass spectrometry" at the FOM Laboratories in Amsterdam, Meuzelaar et al. (3) achieved substantially higher heating rates by means of the Curie point transition in a probe from which a sample is flash-desorbed into the EI source of a quadrupole mass filter. The resulting spectra are usually quite congested and the peaks corresponding to intact ions from very large parent molecules are usually small and rare. Even so, they have yielded much information about composition and identity for substances as complex as coal and bacteria.

A significant advance in heating rate for vaporization and ionization of large molecules also took place in the FOM Laboratories when laser photons were used to flash-desorb molecules from metal surfaces (4). These experiments were forerunners of present day laser desorption (LD) ionization, one of the four "energy sudden" methods that have become widely practiced. The other three include fast atom bombardment (FAB), plasma desorption PD, and secondary ion mass spectrometry (SIMS), which will be referred to here by what we think is a more descriptive and appropriate term, fast ion bombardment (FIB).

All these energy sudden techniques depend upon the nearly instantaneous achievement of a high-energy density in a sample of analyte dispersed on or in a solid or liquid surface. Thus, they can be regarded as perhaps near ultimate extensions of the rapid heating approach originally proposed by Beuhler et al. In FAB the vehicle for energy deposition on the surface is an incident fast atom obtained by charge-exchange neutralization of an ion beam accelerated to several kilovolts. The antecedents of this technique can be found in the early experiments of Devienne and his colleagues (5) with organic molecules on a solid substrate. However, FAB did not become widely used until Barber et al. (6) showed that dispersion of the analyte in a liquid matrix on the surface resulted in better reproducibility and more efficient use of sample. Then continuous FAB was made possible by the development of moving belt systems for transport of sample-bearing matrix into the vacuum system, thus avoiding the inconvenience and low duty cycle inherent in the preparation of individual sample-bearing surfaces for batch-wise insertion (7). Further simplification and convenience have been achieved in "continuous flow FAB" by which a solution of analyte in a liquid matrix enters

the vacuum system through special probes that present a continuously renewed surface to the incident fast atoms. This development was recently reviewed by Caprioli et al. (8).

FIB ionization of molecules in or on surfaces is very similar to FAB in terms of procedures and performance, but evolved from the much earlier development of so-called secondary ion mass spectrometry (SIMS) in which a surface is bombarded by fast incident ions. The original objective of this technique was to produce secondary ions of substrate surface material for mass analysis. Next, Benninghoven and his colleagues (9) found that such ion bombardment could also produce intact ions of large organic molecules deposited on a surface. Since then FIB has enjoyed increasing use in mass spectrometry of complex molecules (10). It is somewhat more convenient than FAB because it eliminates the neutralizing step. Moreover, the incident ion beam can be easily focused on any desired small area of the target surface and can be manipulated so as to scan the surface and map the spatial distribution of the species giving rise to secondary ions. Actually, the distinction between the use of ions and atoms as bombarding species is becoming blurred to the point that the term FAB is sometimes used even when the incident particles are ions.

The earliest and probably the most violent variation on the energy sudden theme is the plasma desorption (PD) technique pioneered by Macfarlane and his collaborators (11). It consists in depositing analyte on the surface of a thin metal foil whose backside is then bombarded by particles resulting from the decay of a radioactive species, usually Californium (^{252}Cf). Its name stems from the fact that the high energy particle released when a Cf atom disintegrates was originally thought to give rise to small blob of plasma on the sample-bearing surface. As many as 30 or more ions are ejected from the impact site when the analyte is dispersed on a matrix of nitrocellulose. Typically the ions are then accelerated through a drift region so that their masses can be determined by time of flight (TOF) analysis for which a convenient zero time is obtained from a backscattered decay product of the Cf disintegration. A small fraction of those ions retain a structure and mass corresponding to the parent species.

In spite of their highly energetic and irreversible nature these energy sudden techniques, LD, FAB, FIB, and PD, are "soft" in the sense that they have been able to produce at least some intact ions of nonvolatile parent species having molecular weights up to several tens of thousands. Results which had been reported at this writing indicate that the record for LD is 210,000 (12), for FAB (or FIB) is 24,000 (13), and for PD is 45,000 (14). The ion currents in most of these experiments have been very small and decrease rapidly with increasing molecular weight. Although parent species having high molecular weights often give rise to ions with up to five or six charges, the charge/mass ratios are generally low. Consequently, when the ions get very large their detection with multipliers requires postacceleration voltages that can be inconveniently high. Moreover, the ions produced have high levels of internal excitation that often results in substantial peak broadening due to predissociation. Indeed, this lack of long-term stability was once believed to a major constraint on the use of Fourier transform ion cyclotron resonance (FTICR) for the mass analysis of large ions from these

sources (15). It is now recognized that the predissociation lifetimes are generally very short relative to the time required for mass analysis by FTICR. Consequently, decomposition of the excited ions may well occur before it can have an appreciable effect on the mass determination.

At first glance, LD would seem to be cut from the same cloth as the other energy sudden methods. However, evidence accumulates that it can be less "catastrophic" and more discriminating in its application of energy than PD, FAB, and FIB. In their landmark experiments Karas and Killenkamp (12) have shown that both the wavelength of the photons and the composition of the matrix in which the analyte is dispersed play a most important role. In particular, a nicotinic acid matrix and an ultraviolet (UV) laser comprise the "magic" combination that has made possible the desorption of proteins with molecular weights over 200,000. Moreover, it appears that the desorbed ions have very little internal excitation.

For purposes of identification and structure elucidation, the large ions that can be produced by these energy sudden methods need the extraordinary capabilities of tandem mass spectrometry (MS-MS) even more than do their smaller cousins. But problems arise in applying MS-MS methods to their mass analysis. As already noted, selected primary ion currents generally decrease as molecular weight increases. Ions from LD and PD appear in short bursts so that time-of-flight (TOF) techniques have been most widely used for mass analysis. Not much experience has been accumulated with MS-MS based on TOF techniques, especially with large molecules, but in our untutored view, obtaining the desired resolution may be difficult. In principle, for MS-MS the combination of FTICR with LD should be attractive because both source and analyzer are batch processes with low duty cycles that, respectively, produce and require only relatively small numbers of ions. Indeed, FTICR offers the intriguing possibility of bringing about in the cell a succession of dissociation and analysis events in a single batch of ions. Unfortunately, the resolution obtainable with FTICR fades rapidly as m/z climbs above 2000 or so. The extent of multiple charging that PD and LD provide does not bring down to this level the m/z values of the large ions they are able to produce.

There is another point to be made in the context of MS-MS for large molecules. When a massive ion collides with a much lighter gas phase atom such as argon, only a small fraction of the former's kinetic energy is available in the center of mass for bond breaking, and that energy can be distributed over many bonds. Therefore, collision-activated dissociation (CAD) for singly charged ions with m/z values above 2500 or so may be difficult to bring about by the usual methods. One possible solution to this problem is to bring about dissociation by photons. Another is to use a solid surface as the collision partner. Such a surface has a very high effective mass so that most of an incident ion's kinetic energy becomes available for breaking bonds. The pioneering studies by Cooks' group (16) indicate that surface targets also have other advantages as collision partners for CAD.

In spite of these problems and difficulties, both real and imagined, the energy sudden techniques have been remarkably effective in extending the advantages of mass spectrometry to the analysis of large and complex molecules. There seems little doubt that they will continue to provide useful results for a long time to come. It will emerge later in this report that ES ionization promises to constitute

an effective complement to PD, LD, FAB, and FIB because it overcomes some of the more refractory problems encountered in their use.

2. Field desorption methods

Quite different in both practice and principle from the ionization methods that we have thus far discussed are techniques that use very strong electrostatic fields to extract ions from a substrate. Two classes of such sources have emerged: those in which the ions desorb directly into vacuum and those in which the desorption is into an ambient bath gas or vapor.

Field desorption into vacuum. Until the introduction of the energy sudden techniques of the preceding discussion, the application of very strong electric fields to samples in vacuo had been the most effective means available for producing intact ions from complex and nonvolatile species. The ionization of molecules near or on sharp points at high potential in vacuo had been discovered in 1951 by Müller (17) and led him to the development of field ion microscopy. The first use of such points as an ion source for mass spectrometry was reported three years later by Inghram and Gomer (18). In 1963 Beckey (19) showed that arrays of "whiskers" on thin wires could produce much higher currents than single needles. He went on to develop what is now known as field desorption (FD) ionization. By its nature FD should do less overt violence to analyte molecules than the energy sudden methods and indeed seems to be softer, probably because it is more reversible. Unfortunately, preparing emitter electrodes, dosing them with sample, and inserting them in the vacuum system, are time-consuming and tedious tasks. Ion currents depend so strongly upon the temperature of the emitter that a deft touch is required to control its heating. Moreover, the ions have several kilovolts of kinetic energy so that magnetic sector instruments have been generally required for their mass analysis. For these reasons FD has not become widely used.

Fields high enough to desorb analyte ions at useful rates from a condensed phase can be achieved without the array of very sharp needles or "whiskers" on a fine wire electrode that Beckey's approach required. Evans and Hendricks (20) found that a high voltage applied to the surface of a nonvolatile liquid in vacuo would produce a sharply pointed "Taylor cone." In a manner analogous to the case of Beckey's FD "whiskers," the field at the tip of this cone was strong enough to desorb ions comprising charge-bearing molecules and clusters of the liquid. When the liquid was a solution, the desorbed ions included solute species. These observations led to so-called electrohydrodynamic (EH) ionization that was introduced to the community of mass spectrometrists by Evans and his associates and has been recently reviewed by Cook (21). EH ionization seems to have been even less widely practiced than its FD cousin, in part because there are very few liquid solvents that can provide the required combination of electrical conductivity and low volatility. A very dilute solution of NaI in glycerol has been often used. Other drawbacks to EH in its present stage of development include the varying degrees of solvation and wide range of energies in the ions it produces. Moreover,

it is prone to cause surface fouling because all of the relatively nonvolatile solvent is dispersed in the vacuum system.

Field desorption into bath gas. A major advance in the production of analyte ions from liquid solutions is embodied in three relatively new techniques that depend upon dispersing analyte solution as a cloud or mist of small charged droplets from which ions desorb into a bath gas rather than into vacuum. In the chronological order of their appearance in the literature these three techniques are electrospray (ES), aerospray (AS), and thermospray (TS). The names are meant to identify them in terms of their most distinguishing operational feature, the method by which they produce charged droplets. In each case the bath gas performs two roles that resolve important difficulties encountered in conventional FD. It provides enthalpy for vaporizing the solvent from the droplets and acts as a moderator to maintain both the internal and translational energies of the desorbed ions at a level corresponding to the bath gas temperature.

Actually, in the chronicle of soft-ionization development the possibility of using charged droplets to produce ions of nonvolatile molecules is not so new. Over 20 years ago, not long after Beckey had started in earnest to develop FD methods and before the energy sudden methods made their debut, Malcolm Dole and his colleagues (1) carried out some pioneering experiments that were pretty much ignored for a long time. Even so, except for the ionization mechanism, the following scenario, much as Dole first described it, still applies to the present practice of all three spray techniques: As the droplets evaporate their surface charge density increases until the Rayleigh limit is reached at which the forces due to electrostatic repulsion approach equality with those due to surface tension. There results an extreme instability, sometimes called a "Coulomb explosion," that produces an array of daughter droplets which also evaporate until they too "explode." Dole's idea was that this sequence would repeat until the ultimate droplets contained only one solute molecule, provided of course that the original solution was sufficiently dilute. As the last of the solvent evaporated from these ultimate droplets, their charge would be retained by the solute molecule to produce a free ion.

This "charged residue" mechanism may actually apply under some conditions, and probably did to some extent in Dole's experiments. Even so, a much more likely explanation for the formation of single ions in the ES, AS and TS sources is field ion desorption as later proposed by Iribarne and Thomson (22). They were concerned with whether the ions produced by vaporization of charged droplets, whose mobilities had been earlier measured by Chapman (23), were charged residues or small individual ions that had somehow evaporated from the liquid. Mobility measurements with higher resolution than Chapman's persuaded them that desorption of small ions did indeed occur. On the basis of a very simple model they estimated values for solvation energies of the ions and calculated the fields necessary for their desorption. They found that for droplets in the right size range, the required fields could be achieved without exceeding the Rayleigh stability limit. Subsequent reports provided mass spectrometric data that seemed to provide evidence of ion desorption for a wide variety of solute species (24).

Thomson and Iribarne nebulized ion-containing solutions to produce the droplets. In such atomization the droplets become charged as a result of statistical fluctuations in the distribution of cations and anions that lead to formation of equal numbers of positively and negatively charged droplets. In their first experiments these authors used only aerodynamic forces, as in a perfume atomizer, to nebulize the liquid. Then they found that 3500 V applied to an "induction electrode" about 1 cm from the atomizing zone produced droplets and ions that were only positive or only negative, depending upon the choice of electrode polarity. Ion currents obtained with this electrode were 100 times greater than could be achieved without it. They called the technique atmospheric pressure ion evaporation (APIE) but we have taken the liberty of dubbing it aerospray (AS). As noted earlier, this convenient and descriptive term stems from the use of aerodynamic forces to produce charged droplets. It thus provides an operational distinction with respect to the other spray sources, thermospray (TS) and electrospray (ES). They also depend upon ion evaporation from charged droplets but produce the droplets by different means. To be completely consistent one should probably identify what Iribarne and Thomson called APIE by a term such as aeroelectrospray (AES). The term aerospray (AS) would then apply when the induction electrode is absent and only aerodynamic forces are at work, as in their first experiments. However, because ion currents are then so much lower, Aerospray is almost never used in that pure form to produce ions for mass analysis. Therefore, it is just as distinctive and much more convenient to use the term AS even when electrostatic augmentation is applied, as it nearly always is in mass spectrometric applications.

Thermospray (TS), the last of the charged droplet sources to be introduced, is by far the most familiar and widely used, in part because it is effective and in part because it is embodied in sources that are available on the market. Developed by Marvin Vestal and his colleagues (25), the TS technique consists in passing the analyte-containing solution through a capillary tube whose walls are hot enough to vaporize 90% or more of the solvent. The resulting expansion produces the same kind of shear and acceleration forces on the liquid that occur in aerodynamic nebulization (as in the perfume atomizer). There results a dispersion of droplets in vapor that emerges from the exit of the capillary as a supersonic jet into ambient solvent vapor at a pressure of 10 to 15 torr in a chamber whose walls are hot enough to maintain the vapor in a superheated state. The already-mentioned statistical fluctuations in the distribution of cations and anions give rise to equal numbers of positively and negatively charged droplets. Evaporation in the superheated vapor brings about the previously described sequence of Coulomb explosions that lead to field desorption of ions from the droplets. The mixture of ions in vapor flows past an aperture through which ions of the desired polarity are driven by an applied field into a vacuum chamber housing a mass analyzer. There is some evidence that an additional contribution to analyte ion formation may result from CI encounters between droplets or vaporized solute molecules and ammonium ions. The latter come from the volatile ammonium salts present as buffers in LC separations when TS is used as an LC-MS interface.

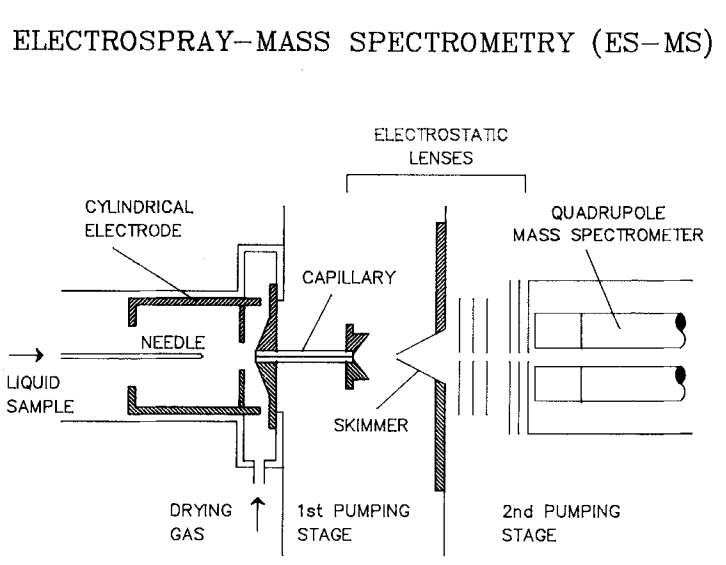

Figure 1. Schematic diagram of an electrospray mass spectrometer apparatus. For details of operation see text.

III. THE ABC'S OF ESMS TECHNOLOGY

A. Apparatus and procedures

Figure 1 shows a schematic representation of an apparatus in our laboratory that embodies the essential features of electrospray mass spectrometry (ESMS) and has been described elsewhere (26). The following account of its operation attempts to provide a convenient introduction to its principles. Sample solution at flow rates usually between 1 and 40 μL/min enters the ES chamber through a stainless steel hypodermic needle at ground potential. When it is desired to maintain the source of sample liquid at ground potential (e.g., in LC-MS), values of applied voltages that are typical appear in parentheses after each of the following components: needle (ground), surrounding cylindrical electrode (−3500), metalized inlet and exit ends of the glass capillary (−4500 and +40, respectively), skimmer (+20), ion lens in front of quadrupole (ground). To produce negative ions similar voltages of opposite sign are applied. In addition, it is useful to introduce a small stream of oxygen or other electron scavenger near the needle tip to inhibit the onset of the corona discharge which occurs at lower voltages in the negative ion mode (27). Such coronas can be seen if the room is sufficiently dark and heard if it is sufficiently quiet. When the voltage is high enough to produce a corona in the positive mode the adduct cation in the spectral peaks generally becomes a proton, no matter what it was at lower voltages (27). In the negative mode the spectra sometimes contain peaks for ions to be expected if there is a discharge in a gas containing O, N, and C (i.e., O^-, NO^-, CN^-, NO_2^-, OCN^-, and O_2^-).

At first glance, the indicated potential difference of 4540 V between the inlet and exit ends of the capillary may seem startling. We have found that with the

carrier bath gas (nitrogen) at about one atmosphere, the ion mobility is low enough so that the gas flow through the capillary can drag the ions out of the potential well at the capillary inlet and raise them back up to ground potential or as much as 15 keV above it (28). Thus, we can readily provide the energies necessary for injection into a magnetic sector analyzer. The capillary, with a bore of 0.2 × 70 mm, passes just about the same flux of both bath gas and ions as did the thin plate orifice (d = 0.1 mm) that produced the free jet "lock" between high and low pressure chambers in our first apparatus (27). In the new configuration the sample injection line along with all other external parts of the apparatus are at ground potential and do not pose the hazard that is generally present when the glass capillary or its equivalent is not used.

The field at the needle tip charges the surface of the emerging liquid, dispersing it by Coulomb forces into a fine spray of charged droplets. Driven by the electric field the droplets migrate toward the inlet end of the capillary through a countercurrent flow of bath gas typically at 800 torr, an entering temperature from 320 to 350K, and a flow rate of about 100 mL/s. The solvent vapor from the evaporating droplets along with any other uncharged material is swept away from the capillary inlet by the bath gas flow. Meanwhile, in accordance with the scenario described earlier, the rapid evaporation of the migrating droplets promotes the sequence of Coulomb explosions that gives rise to droplets with a radius of curvature so small that the electric field at their surface is high enough to desorb solute ions into the ambient gas. Even solute species that are not ionic can attach solute cations or anions to their polar groups and desorb from the droplet as so-called "quasimolecular ions" suitable for mass analysis. Some of these desorbed ions are entrained in the flow of dry bath gas that enters the glass capillary to emerge at the exit end as a supersonic free jet in the first of two vacuum chambers. A core portion of this free jet passes through a skimmer into a second vacuum chamber, delivering ions to the analyzer which in our present system is a quadrupole mass filter (VG Micromass 1212) with a nominal upper limit for m/z of 1500.

B. Further reflections on Dole's experiments

The apparatus and procedures in Dole's original experiments were similar to those just described except for some noteworthy differences. Because he was concerned with macroions whose masses were larger than could be accommodated by analyzers then available Dole resorted to energy analysis for mass determination. Ion energy E was obtained by measuring V_r, the voltage on a grid just sufficient to prevent the ion from passing through to a Faraday cup detector. Ion velocity v was assumed equal to the readily calculable jet gas velocity. From $E = mv^2/2 = zV_r, m/z$ was then readily obtained.

In addition to the horizontal mode of Figure 1, Dole tried vertical geometries with the electrospray needle pointing both up and down. The latter was convenient for assembly and access but gave trouble because occasional large droplets fell into and plugged the nozzle. Also to be noted is that Dole's ES chamber comprised a glass cylinder, 13 cm in diameter and 36 cm long, in which charge

accumulation on the walls could have produced some observed effects that seem somewhat anomalous. For example, it was found that with grounded aluminum sheet wrapped around the outside of the chamber the transmitted ion currents were somewhat larger, reached a steady state more quickly, and were less sensitive to the radial position of the liquid injection needle. Our ES chamber has metal walls and is much smaller: 5 cm in diameter and 15 cm in length. Occasionally, after days of operation with liquid flow rates, solute molecular weights and/or solute concentrations that are larger than usual, we have also encountered decays in observed ion current. We attribute these decays to fouling and consequent charging of exposed surfaces because the current can be restored to initial levels simply by cleaning those surfaces.

To indicate other differences between the two sets of experiments, the pair of numbers immediately after each of the following operating variables are typical optimum values, the first for Dole's experiments, the second for ours: sample liquid flow rate: 80 versus 10 μL/min; bath gas flow rate: 7 versus 6 L/min; distance and potential difference between liquid injection needle tip and end plate of ES chamber (exit plane for gas entering vacuum system): 21 versus 2 cm and 15 versus 4.5 kV. The biggest of these differences clearly have to do with the distance and voltage drop between the liquid injection needle and the end plane of the ES chamber. The larger separation distance would clearly require a higher voltage to provide the field necessary for electrospray dispersion of the entering liquid. This larger separation distance may also account for the larger value of optimum liquid flow rate in Dole's experiments.

A key question seems to be: why was Dole's optimum separation between injection needle and end plate so large? We think the main reason is that his bath gas flow was concurrent with the drift of charged droplets so that more time (distance) was needed for their complete evaporation than in our experiments where the gas flow was countercurrent to the drift direction. Indeed, with concurrent flow it seems highly unlikely that all solvent vapor could have been removed from the bath gas arriving at the end plate of the ES chamber. Consequently, some of Dole's observations could have been affected by condensation of vapor on the ions during free jet expansion.

At this point it seems appropriate to consider some aspects of the question as to when and/or whether the Dole charged-residue mechanism may apply to the production of free ions from charged droplets. In the first place we note that it is difficult to imagine a combination of charge and mass in a parent droplet that could by Coulomb explosion produce the singly charged droplets that the Dole mechanism requires to explain his apparent observation of singly charged macroions. This possibility seems especially remote in view of the available data indicating that the charge/mass ratios of the smaller product droplets in such explosions are higher than for either the parent droplet or its larger progeny (29). In the second place, as Dole himself recognized, the flux of analyte molecules in the electrosprayed solution was 10 to 100 times greater than the flux of unit charges. Consequently, it is hard to understand how his ultimate droplet could contain only one molecule even if it did retain only one charge. A third problem is posed by his assumption that during the free jet expansion into the vacuum

system the macroions reached the same velocity as the carrier gas molecules. There is a substantial body of evidence, both experimental and theoretical, that large molecules encounter slip effects during free jet expansion of neutral gas mixtures at the nozzle Reynolds numbers used in Dole's experiments. Major velocity lags have been observed for heavy species in free jet expansion of neutral gas mixtures for which the mass ratio of heavy to light components is 100 or more times smaller than in the mixtures he used (30). It seems unlikely that the charge on a macroion could increase its effective drag coefficient enough to overcome the slip that it would experience if it were neutral.

Finally, we note that in numerous experiments with solutions of various hydrocarbon molecules (including styrene), having masses up to several hundred daltons but well within the range of our quadrupole analyzer (1500 Da), we have never been able to detect ions that we could attribute to the desorption of hydrocarbon solute species from the charged droplets in accordance with the Iribarne–Thomson model. Such ion formation from species that are not themselves ions always seems to require the presence of a polar atom or group to which solute cations or anions can be rather strongly bound by ion–dipole forces. Moreover, as we will show in later discussion, analyte ions with masses above some critical value require more than one charge to be desorbed from a droplet. This critical mass depends upon the nature of both solvent and solute and is usually between about 1000 and 2000 daltons. The number of charges required for desorption increases with mass in such a way that m/z always seems to be less than about 2000.

In sum, we do not think that singly charged macromolecules of any of the polymers in Dole's experiments (polystyrene, the protein zein, and polyvinylpyrrolidone) could possibly have been formed by his charged-residue mechanism. Nor, in the case of polystyrene, could any ions have been formed by the desorption mechanism of Iribarne and Thomson. Moreover, even if such singly charged ions had been formed by some mechanism, they could not have been accelerated to the calculated gas velocity in the free jet. Consequently, we think the most likely explanation for Dole's observations is that his ultimate droplets contained a plurality of both solute macroions and charges. Thus, after all solvent had evaporated the residue comprised clusters of solute molecules with a number of attached charges. Some of these aggregates had just enough charge so that at the actual velocity reached during free jet expansion their kinetic energies corresponded to what would be expected for singly charged molecules at the calculated velocity of the carrier gas. Similar "coincidences" could account for the structure observed at higher retarding potentials in some of Dole's current-voltage curves. This speculation seems consistent with his finding that in different current-voltage curves for the same analyte the steps sometimes occurred at different voltages.

It should also be said that the mechanism proposed by Iribarne and Thomson is not unquestioned. Using their estimates of solvation energies Röllgen's group (31) has argued that field desorption, as described by Iribarne and Thomson, cannot play an important role in ion formation from discharged droplets. They suggest that extremely small droplets containing only one solute molecule may be ejected during the instability behaviour (Coulomb explosion) that occurs at the

Raleigh limit when surface tension and Coulomb forces are of the same order. Although this case against ion production by evaporation seems plausible, we find it hard to understand how even strong electrohydrodynamic effects can account for the results that we obtain with solutions containing proteins and other polymers. Later in this report we will present those results and discuss their implications.

This somewhat speculative reinterpretation of Dole's results does not in any way detract from the significance and importance of his contribution. Even though his ideas on mechanism seem to require some modification, he has earned abiding recognition for daring to believe that very large molecules might become accessible to mass spectrometric analysis and for being the first to recognize what might be achieved by combining free jet molecular beam techniques with electrospray dispersion of analyte solutions into a bath gas. Fully as praiseworthy are Dole's ingenious and provocative experiments. They blazed a trail that has led to rewarding results for those of us who have followed in his footsteps.

C. Results with smaller solute species

When Dole's first reports appeared, our laboratory had been having a ten-year love affair with supersonic free jets as molecular beam sources. Consequently, we were sufficiently intrigued with his results to carry out some experiments that confirmed his observations. Then he and we both abandoned further efforts, in part because of velocity slip and other problems with retarding potential mass determination, and in part because the primary ion currents were very small and could not be enhanced by the millionfold gain one comes to expect with multiplier detectors. Singly charged ions as large as we then thought we were forming would not produce secondary electrons by colliding with a surface unless, as Beuhler and Friedman later reported (32), they were first accelerated to half a million volts or so.

In 1980 we went back to ES experiments, this time with solutions of solutes having low molecular weights as well as low volatilities. The apparatus was very similar to that in Figure 1, except that ion-bearing gas entered the vacuum system through a simple flat plate orifice instead of the glass capillary, and the quadrupole had an upper mass limit of only 450 Da. The idea was that we might learn something about the assumed sequence of Coulomb explosions from the effects of operating variables such as solute concentration, solvent composition, applied voltage, and bath gas flow, on the masses of the ion clusters we expected to observe. Unaware at that time of the earlier results of Iribarne and Thomson with AS, or those of Vestal and his colleagues with TS, we still believed in Dole's charged-residue scenario.

We soon found that the ions we observed comprised adducts of solute cations or anions with one or more solvent and/or solute molecules that were not themselves ions. If the temperature and flow rate of bath gas were high enough, the solute concentration low enough, and the solvent sufficiently volatile, the only significant peaks in the observed mass spectra were those due to adducts of single nonionic solute molecules with anions or cations from the solution (27). We went

on to try a wide range of solute species including alcohols, acids, esters, amines, catechol amines, cholines, nucleotides, amino acids, and small peptides. In every case the spectra comprised peaks for the solute species itself when it was an ion or for its adducts with anions or cations when it was not an ion. Although occasionally there were additional peaks due to solvation of solute ions, including those that were adducts with nonionic parents, the spectra were remarkably uncongested and never showed any evidence of parent species fragmentation. Our quadrupole's stability, resolution, and transmission characteristics left much to be desired so we did not attempt any quantitative determinations of sensitivity. Even so, with solute concentrations in the ppm range, simple single mass scans on a chart recorder with no signal averaging provided clean spectra with high signal/noise. Figure 2(a), for example, shows the positive ion spectrum obtained with a mixture of one quarternary phosphonium and six quaternary ammonium halides at concentrations ranging from 3 to 10 ppm in 50–50 methanol water. The spectrum of Figure 2(b) shows clearly separated peaks for intact ions of each species in a vitamin B tablet that has a molecular weight below 450, the upper limit of our quadrupole.

These particular spectra are shown as examples because they clearly indicate the ability of ES ionization to avoid fragmentation as well as interference between coexisting species. Another reason is what they have to say about the mechanism of ion formation. In these early experiments, as well as in Dole's, there were always many more analyte molecules than charges in the ES flux. Consequently, as the results accumulated we had become increasingly uneasy about the applicability of his charged-residue model. The spectra in Figure 2 heightened that concern because we could not understand how the sequence of Coulomb explosions could produce, for each of several species in the original solution, singly charged ultimate droplets containing only one molecule of that species. Moreover, the relative numbers of those droplets for each species would have had to be in direct proportion to the relative concentration of those species.

We were rescued from these difficulties by discovering the Iribarne–Thomson papers that revealed the more credible desorption mechanism for ion formation and presented results from their experiments with AS ionization. At about that same time we learned about the TS ionization experiments of Vestal and his colleagues. Similarities in the mass spectra obtained by all three of these "spray" techniques increased our confidence in the ES results as well as in the general applicability of the ion-desorption model for ion production from charged droplets in a bath gas.

It is now appropriate to remark on some of the differences and similarities between these techniques. The a priori distinctions between the AS, TS, and ES sources are primarily operational and relate mainly to the method of producing charged droplets. In AS and TS, charging is brought about by atomizing an ion-bearing liquid, statistical fluctuations in the distribution of cations and anions among the droplets accounting for their charge. In ES, atomization is brought about by charging the surface of the liquid. Thus, ES can in some sense be considered as a mirror image of AS and TS. Consequent to these operational distinctions are some important differences in performance. AS and TS work best

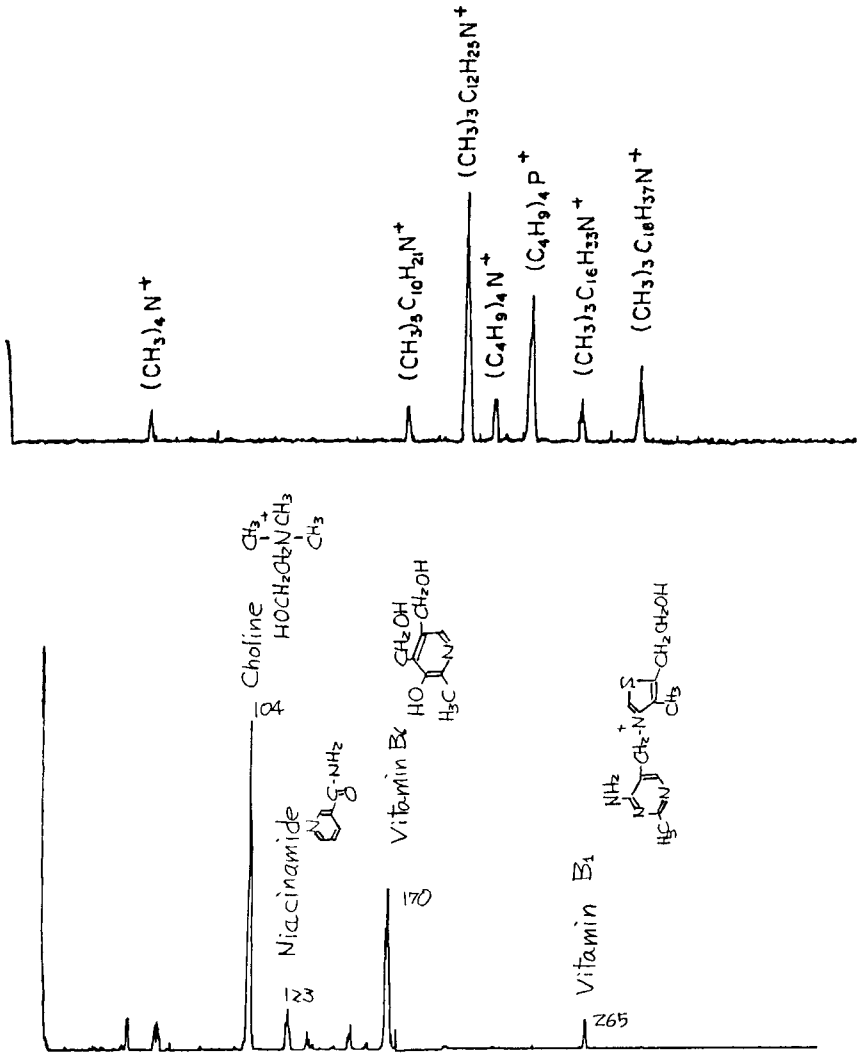

Figure 2. Typical electrospray spectra for nonvolatile small molecules: (a) For a mixture of quaternary ammonium and phosphonium halides at concentrations from 2 to 10 ppm in 50:50 methanol-water. (b) For an extract of a vitamin B tablet in 50:50 methanol–water.

with flow rates in the 0.5 to 2 mL/min range encountered in conventional liquid chromatography (LC). ES prefers flow rates from 1 to 40 μL/min and thus can work with smaller amounts of analyte. These low flow rates seem better matched to LC on the microbore scale which is growing in popularity. They are also well adapted to the very low flow rates encountered in capillary zone electrophoresis (CZE) which is now attracting a lot of attention because of its very high separating efficiency, equivalent to a million theoretical plates in some experiments. Indeed, an ES mass spectrometer seems to be an exceedingly promising detector for CZE

separations (33). ES dispersion does not yet work well with liquids having electrical conductivities higher than those of 0.005 *M* KCl solutions. AS and TS have no such difficulties until much higher ionic solute concentrations are reached. Smith et al. (34) have found that by providing an annular sheath flow of pure solvent around the analyte-containing core flow, successful ES dispersion can be achieved when the core flow by itself could not be so dispersed because conductivity and surface tension have unfavorable values. AS and TS seem to encounter no such dispersion difficulties.

All three methods have shown themselves capable of producing quasimolecular ions in vacuo from a wide variety of solutes of biochemical interest including peptides and polypeptides, amino acids, sugars, nucleotides, and nucleosides. In the relatively few experiments that offer a basis for direct comparison, ES has shown somewhat greater sensitivity, in the attomol range for small proteins and peptides, and can accommodate to smaller samples than can AS and TS. There is fragmentary anecdotal evidence that ES works better than TS with some substances and that it may be somewhat softer for labile species than TS which perforce exposes samples to higher temperatures. For example, sulfonated azo dyes that did not produce ions by TS were readily ionized by AS and by ES (35b). We have obtained spectra of unfragmented ions for a number of species that have been sent to us by analysts who had tried TS without success. Unfortunately, there have been no definitive comparisons of the three techniques for relative "softness" or "effectiveness" under controlled conditions by operators equally skilled in the art of each technique.

It is in order to comment further on combinations of thermal, aerodynamic, and electrostatic forces in the production of charged droplets. Dole's second article reports having passed the bath gas flow through the annular space between the liquid injection capillary and an outer concentric jacket (1). The resulting high velocity flow of bath gas over the capillary tip was meant to assist in the atomization of the emerging liquid but seemed to produce larger droplets and a smaller ion current than atomization by the electric field alone. Coming later from the other direction to the same combination, as noted earlier, Thomson and Iribarne obtained much higher currents than with aerodynamic atomization alone when they applied an electric field by placing a 3 keV "polarizing electrode" close to the intersection of the streams of sample liquid and atomizing air. They also found that large droplets were produced so they passed the droplet-laden gas around a right angle bend to remove them by inertial effects (24).

In sum, charged droplets can be produced thermally, aerodynamically, and electrically, separately as in TS, pure AS, and ES or by some combination of these. Recently, Bruins et al. (35a) rediscovered the combination of aerodynamic and electrostatic forces using the same configuration tried originally by Dole. They call it "Ionspray," a term which has now been adapted as a trademark by Sciex. In our view this development is unfortunate, because the term is neither needed nor is it meaningfully descriptive. (As we have noted earlier, inapt and inept nomenclature occurs too often with the result that inappropriate names become established in the literature and remain a source of awkwardness and confusion as, for example, in the cases of SIMS and APIE.) Bruins et al. (35a) point out, as

was already known from the AS experience of Thomson and Iribarne (24), that the aerodynamic assist to ES allows the use of higher liquid flow rates and can atomize solutions of higher conductivity than can unassisted ES. They also provide some welcome information on comparative droplet sizes. Initial droplet diameters in pure ES dispersion are the smallest (1–2 μm) and TS droplets are the largest (>100 μm). Their ionspray combination of aerodynamic and electrostatic forces provides droplets of the same size (12 μm) as does the AS of Thomson and Iribarne (which Bruins et al. refer to as "ion evaporation"), thus indicating the fundamental equivalence of the two. More recently, Henion (36) reported that ES and ionspray give about the same mean droplet size of around 10 μm. (This value seems too large to account for the second order Tyndall spectra that are readily observed in bench experiments with ES dispersion.) Henion's group (36) has also looked at thermally assisted electrospray and finds that it allows operation at higher flow rates than ES alone, but that sensitivity and multiple charging are appreciably reduced.

From the fragmentary data available on ion currents and sample flow rates we conclude that the ratio of charge to analyte in droplets from pure ES is always higher than in droplets produced by TS, AS, or by any combination of thermal, aerodynamic, and electrostatic forces, by whatever name. This combination of small size and high charge/mass ratio in ES droplets is probably responsible for the high detection sensitivity provided by ES ionization. It almost certainly accounts for the extensive formation of multiply charged ions that will be discussed in the next section. What seems to be emerging from accumulating experience is what one should intuitively expect: the name of the spray game in ionization is charge/mass ratio, the higher the better. Another emerging implication is that any attempt to "assist" ES inevitably decreases the charge/mass ratio. If such assistance is to decrease the ES contribution to the dispersive work requirement, it must ipso facto decrease the charge density on the surface of the emerging liquid. It appears that the only practical advantage of these combination techniques is to allow the use of higher flow rates. Because they also decrease sensitivity and the extent of multiple charging (usually a substantial advantage as later discussion will show), effective flow splitting would be a much more attractive approach to the flow rate problem. It would also save sample for other uses.

IV. LARGE SPECIES AND MULTIPLE CHARGING

Interested in our early ESMS results with small molecules, VG Analytics, Ltd. kindly loaned us a quadrupole mass filter (VG MM 1212) that could analyze ions with m/z values up to 1500, substantially above the limit of 450 in our original instrument. We then assembled the apparatus shown in Figure 1, whose operation was detailed in the last section. Some preliminary shakedown experiments gave results with species such as adenosine and its mono- and diphosphates equivalent to those obtained with the first machine. Then we tried our luck with larger species, some peptides with molecular weights between 1000 and 1500. Figure 3(a) shows a spectrum obtained with the antilymphocytic agent cyclosporin A for

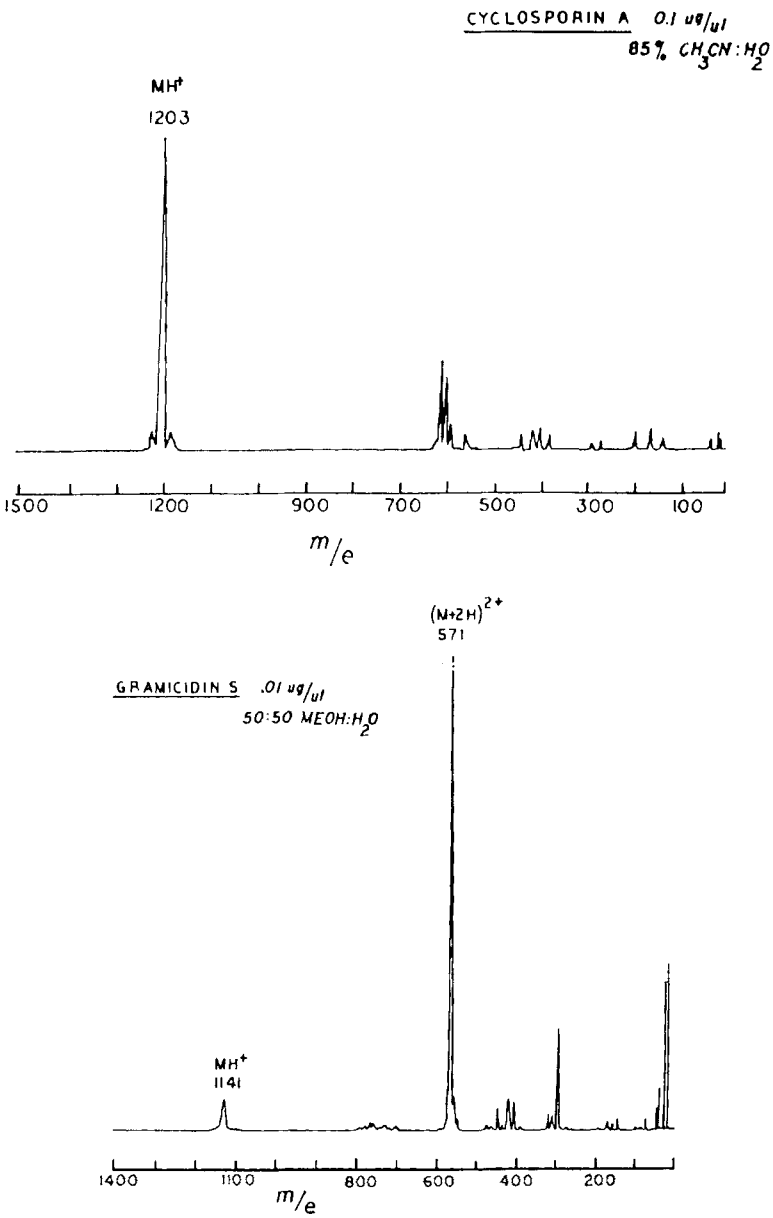

Figure 3. Electrospray spectra for two peptides: (a) Cyclosporin A in acetonitrile–water; (b) Gramicidin S in methanol–water.

which the dominant peak at $m/z = 1203$ corresponds to a singly protonated parent molecule with one water molecule of solvation. The small satellite peaks on either side, respectively, represent ions with one more and one less molecule of solvation. The much smaller triplet peak near mass 600 comprises the same ions as in the large triplet except that they are doubly protonated. The other small peaks at the low end of the m/z scale stem from impurities or solvated trace ions. There

is no evidence of parent molecule fragmentation due to the ionization process in these spectra or in any others that we have obtained.

Figure 3(b) shows a spectrum for another peptide, gramicidin S. It is similar to the one for cyclosporin A except that the peak for doubly protonated parent is much larger than the one for singly charged ions. In similar spectra for the peptides bleomycin and "substance P" the doubly charged peaks also predominate. In the spectra for renin substrate and insulin B chain there is evidence of triple protonation. This propensity for multiple charging is quite provocative because the mass range of any analyzer is increased by a factor equal to the number of charges per ion.

A. Results with poly (ethylene glycols)

In order to explore the extent to which multiple charging can occur, as well as to identify and evaluate the controlling factors, we carried out an extensive study of ES ion formation with poly(ethylene glycol) (PEG) oligomers (37). These species retain their chemical and structural similarity over a wide range of molecular weights. Samples, readily available from a manufacturer (Union Carbide Chemical), comprise a mixture of oligomers with a Gaussian distribution of molecular weights having a FWHM roughly 15% of the molecular weight of the most abundant oligomer, the nominal molecular weight of the sample. The mass difference of 44 Da between successive oligomers is large enough so that, within limits to be discussed, only modest resolution is required to distinguish between peaks for particular oligomers even when each ion has several charges. In Figure 4(a), the ES mass spectra of PEG with a nominal molecular weight of 400 shows a Gaussian distribution of predominant peaks corresponding to oligomer ions whose single charge is an Na^+ from sodium in the sample. When the spectrum is enlarged, a similar distribution of much smaller subpeaks due to oligomers with K^+ as an adduct can be discerned. A third distribution of still smaller peaks indicates oligomers to which two Na^+ are attached. Addition of K^+ (e.g., as KCl) to the initial solution makes the K^+ peaks predominant. Additions of NaOH substantially increase the peak intensities for Na^+ adducts.

As the nominal molecular weight of the sample goes up, the number of Na^+ adducts per oligomer in the predominant peaks also increases. This trend is revealed in Figure 4(b–d) that, respectively, show spectra for PEG samples with nominal molecular weights of 1000, 1450, and 3350. A Roman numeral indicates the number of charges per oligomer in the associated band of peaks. In the spectrum of Figure 4(d), for which the nominal molecular weight has reached 3350, the singly charged band has disappeared (in the range of m/z below 1500), and the predominant peaks are for quadruply charged oligomers. On an expanded scale, the two bands labeled V and VI clearly show that component peaks are for oligomers with 5 and 6 charges. At first glance these changes in PEG spectra with increasing molecular might seem unreasonably dramatic. In fact, they are entirely consistent with a model for charge limitation that we are about to discuss. Here we will simply note that the result of increasing the molecular weight from 1000 in Figure 4(c) to 1450 in Figure 4(d), for example, is a large fractional increase in

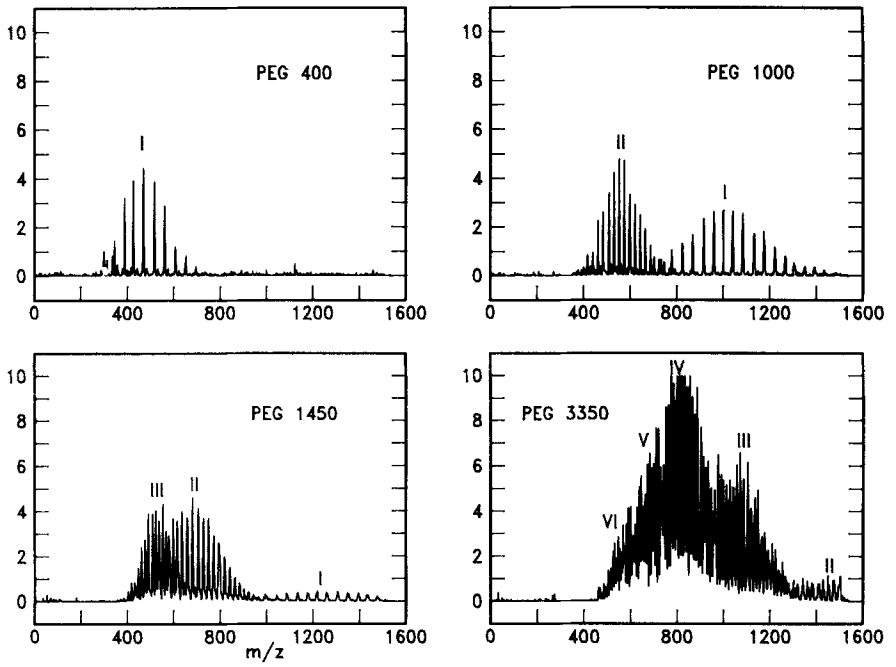

Figure 4. Electrospray spectra for poly (ethylene glycol) samples of varying molecular weight in methanol–water. The nominal molecular weight refers to the most abundant oligomer.

the number of molecules big enough to retain three charges. At the same time, there is a large fractional decrease in the number of molecules so small that they can retain only one charge. It is this "double-barrelled" effect that makes such pronounced differences in the spectra for these two samples.

Both the congestion and jaggedness of profile in the spectral bands of Figure 4 clearly increase as the nominal molecular weight of the PEG sample and the number of charges per oligomer both increase. These effects stem from the associated increase in the number of possible combinations of oligomer mass and charge which lead to increasing congestion and superposition of peaks too close together on the *m/z* scale to be resolved by our analyzer. For PEG 8000 and 17,500 the spectral congestion is such that there are as many as 6 or 7 peaks per unit interval in *m/z* so that the spectra comprise bands with nearly continuous but noisy-looking profiles as shown in Figure 5.

In spite of their lack of resolution these spectra have interesting features that provide valuable information. If we assume that the *m/z* value of the profile peak in each spectrum corresponds to the most abundant oligomer with a most probable number of charges, we arrive at values for that number of 10 and 23, respectively, for PEG 8000 and 17,500. Although this conclusion, as presented, may appear to be based on fairly bold inference, its credibility will be supported in later discussion of results with peptides and proteins having even higher molecular weights.

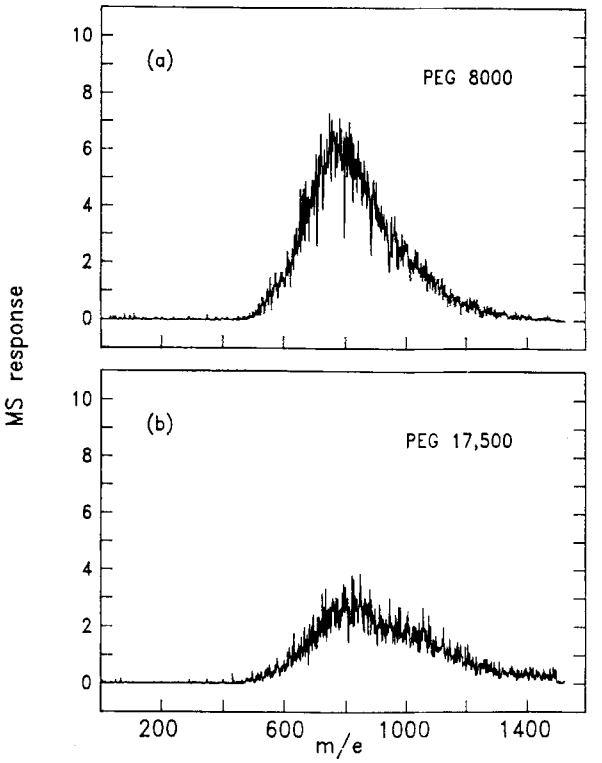

Figure 5. Electrospray spectra for poly (ethylene glycol) samples of "high" molecular weight in methanol–water. The most abundant oligomer in the "20*M*" sample in 17,500.

A question naturally arises: How many charges can there be on an ion and what determines that number? One limiting factor, the maximum number of charges a free molecule can retain, is reached from the following assumptions: (1) the cations, Na^+ in these experiments, bind to the O-atoms in the PEG molecules; (2) they are distributed at equidistant intervals along the length of the oligomer chain that, because of electrostatic repulsions between the charges, is in its stretched-out "zig-zag" configuration; (3) the centermost charge has the highest electrostatic potential energy. That energy can be calculated by pairwise addition of the repulsion terms due to interaction with the other charges on the chain. The pairwise distances are determined from the first two assumptions in light of the known geometry of the PEG oligomers. The number of charges in this linear array is maximum when the electrostatic potential energy of the centermost charge just equals the binding energy between that charge and its O atom. That binding energy for Na^+ in PEG is assumed equal to the measured value of 2.05 eV for the bond between Na^+ and the O atom in dimethoxy ethane which is chemically similar to an O atom in PEG.

This model gives rise to the curve in Figure 6 for the relation between the size

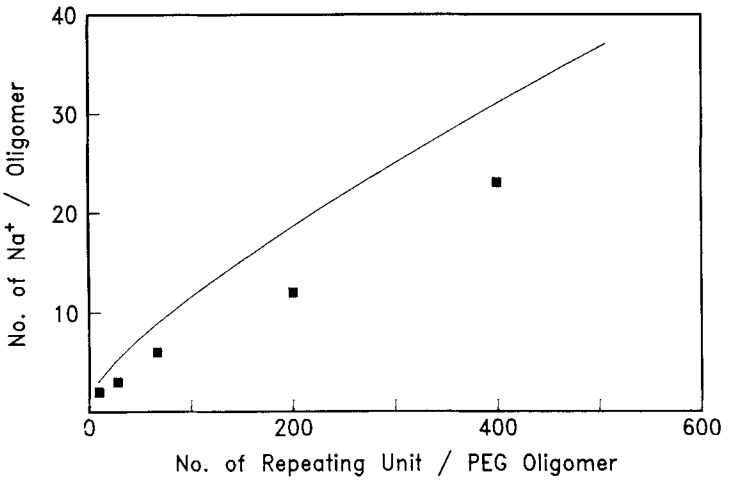

Figure 6. Dependence of the number of charges (Na^+) per oligomer of poly (ethylene glycol) on the number of constituent monomers. The solid curve shows what the model predicts. The squares are from measured spectra.

of an oligomer and the number of charges. The points represent experimental values obtained from measured mass spectra. Clearly, the model allows more charges per oligomer than are realized in the experiment. Part of the discrepancy may be due to factors that it ignores. For example, fluctuations in oligomer configuration due to Brownian motion may result in an average distance between O atoms that is less than the distance based on the assumption of a linear stretched configuration. Shielding effects due to the oligomer structure and solvent interactions may attenuate the Coulombic repulsion forces between the charges and/or the charge-dipole attractive forces between the Na^+ ions and the O atoms. It seems more likely, however, that the discrepancy is kinetic rather than thermodynamic in origin. This possibility will be examined after results with other kinds of large molecules are discussed in the next section.

B. Results with small proteins

Some of the congestion problems encountered with PEGs, and other polymer samples that are mixtures of oligomers, would be avoided by the use of analyte species that have a high molecular weight but are pure compounds. So called biopolymers like proteins and nucleic acids are examples of such species and can be readily obtained over a wide range of molecular weights. A more important reason for experiments with these materials is the growing interest in the prospective role of mass spectrometry in determining their identity and structure (38). Results recently obtained with ESMS of small proteins will now be summarized briefly (39).

Effective solvents comprised mixtures of acetonitrile, water, and methanol or l-propanol, with small additions of acetic acid (HAc) or trifluoroacetic acid (TFA). Solutions with analyte concentrations ranging from 0.7 to 137 μmol/L were in-

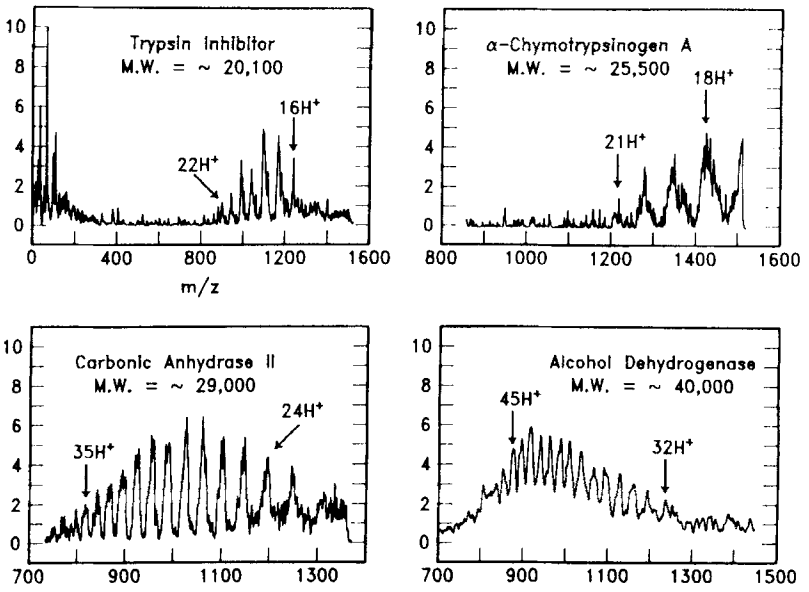

Figure 7. Representative electrospray mass spectra for protein samples in acidified mixtures of water, methanol, and isopropanol. The adduct ions were protons. Solution concentration varied from 5 to 19 μmol/L and injection was at 8 μL/min. Each spectrum resulted from a single 30-s scan of the indicated mass range. The charged species were proton.

jected at rates of 8 μL/min. Spectra for each of eight proteins with molecular weights between 5,000 and 40,000 were obtained from a single 30-s scan for the indicated mass range. Figure 7 shows representative mass spectra for four of these proteins. Each spectrum comprises a sequence of peaks each of whose ions differ from those of the adjacent peaks by one charge. To provide a frame of reference the number of charges per ion is shown for two or three peaks in each case. Although the resolution of our analyzer does not provide unequivocal confirmation, we obtain reasonable results by assuming that each adduct charge is a proton.

Table I summarizes essential features of the spectra for all eight proteins studied, along with the information they provide. Immediately apparent from the figures and the table is the ability of ES ionization to achieve multiple charging to a much greater degree than has yet been possible with any of the other "soft" ionization techniques. Such multiple charging is most attractive because it increases the nominal mass range of any analyzer by a factor equalling the number of charges per ion. Thus, in these experiments ions having masses up to 40 kDa could be "weighed" with an analyzer whose nominal upper mass limit was 1500 Da.

As in the case of PEG the number of charges per ion increases in approximate proportion to the molecular weight of the parent species, reaching a value of at least 46 in the case of alcohol dehydrogenase. However, it is not yet possible to calculate the maximum number of charges that can be retained by one of these protein molecules. The electrostatic potential model used to calculate this maxi-

Table I. Summary of Molecular Weight Results for Protein Spectra

Mol. weights[a]	From sequence M_s	Unweighted average M_r	$\Delta M_r(\%)$	Weighted ave., $p = 2$ $M_{w,2}$	$\Delta M_{w,2}(\%)$	By deconvolution M_d	$\Delta M_d(\%)$
Insulin	5733	5734 ± 14[b]	0.01	5740 ± 12	0.12	5751	0.31
Cytochrome C	12,360	12,349 ± 5	0.09	12,350 ± 4	0.08	12,352	0.07
Lysozyme	14,306	14,324 ± 15	0.13	14,329 ± 10	0.16	14,340	0.24
Myoglobin	16,950	16,906 ± 11	0.26	16,904 ± 7	0.27	16,927	0.26
Trypsin Inhibitor	20,091[c]	19,990 ± 37	0.50[c]	20,001 ± 28	0.45[c]	20,023	0.34[c]
α-Chimotrypsinogen A	25,656[d]	26,131 ± 22	1.8[d]	26,130 ± 22	1.8[d]	25,939	1.1[d]
Carbonic Anhydrase II	29,006	28,982 ± 16	0.08	28,984 ± 12	0.08	29,005	0.004
Alcohol Dehydrogenase	39,830	39,859 ± 25	0.07	39,871 ± 17	0.10	39,876	0.12

[a]All molecular weights are isotope averaged.

[b]Standard Deviation in the averaged values of molecular weights from the individual peaks.

[c]75% of the molecules have been reported to lack the terminal Leu, which if true in our sample would lower the average mass and lead to better agreement with the measurement.

[d]This may be an atypical case since there are only four broad peaks to average and the mass window extends beyond m/z = 1500 (see Fig. 2). However, the standard deviation indicates that the measurement error in this spectrum should be only slightly higher than in the other spectra.

mum number for PEG requires information on structure, configuration, binding site, and binding energy that is not available for these much more complex molecules. Indeed, vice versa, observed ratios of charge to mass may provide some insight on the configuration of biopolymers. For example, Mann (40) in our laboratory found that the maximum m/z values (minimum number of charges) for proteins of similar molecular weight increase as the number of disulfide bonds increases. This observation is consistent with the idea that disulfide bonds prevent the molecules from stretching out. Thus disulfide bonds decrease the distance between prospective charge-bearing sites. It follows that the electrostatic repulsion force felt by each charge is higher per attached charge for ions constrained to a compact configuration than it is for ions that are free to unfold and stretch out. Therefore, such compact molecules cannot retain as many charges so their ions have higher m/z values.

C. Some implications of the ES spectra for PEGs and proteins

As Figure 5 shows, individual oligomer ion peaks cannot be resolved in spectra for samples of PEGs having nominal molecular weights comparable to the subject proteins (i.e., PEGs 8000 and 17,500). Consequently, it is not possible to distinguish the distribution of peak amplitudes for ions comprising a particular oligomer with differing numbers of charges. In the spectra for proteins, on the other hand, the peaks for ions corresponding to a particular parent molecule with differing numbers of adduct charges are clearly resolved. Even so, the envelopes of these

protein ion peaks and the profiles of the PEG ion "bands" both have a similar Gaussian shape, and in each case define an ion desorption "window" in the *m/z* scale. The approach to the fairly distinct lower boundary of this window is rather steep in terms of decreasing peak height. The less distinct upper limit to *m/z* is approached at a more gradual rate of decrease in peak height. These differences in shape at the upper and lower ends of the window are due in part to the fact that the *m/z* scale is not linear in the number of charges. For an abscissa scale in terms of *z/m*, the shapes of the envelopes are in general more symmetrically Gaussian.

We speculate that these window limits may be explained by the strong dependence of ion desorption rate on droplet surface charge density. Clearly, that rate should be proportional to the number of charges per ion and the magnitude of the electrostatic field at the surface. Both of these quantities increase with increasing density of charge at the droplet surface. Thus, the observed ions with the lowest number of charges (at the highest value of *m/z*) are those whose desorption rate has become just large enough to result in a detectable ion current. As evaporation increases the surface charge density, both the field and the number of charges per ion increase at the surface so the desorption rate increases rapidly. Consequently, the ion current (peak height) must increase with increasing number of charges/ion (decreasing *m/z*) as the spectra show. This trend continues until the desorption rate of ions with a particular *m/z* becomes so high that the residence time of an ion on the surface is of the same order as the time required for it to achieve the charge state (collect all the charges) that would be possible at the existing surface charge density. The net result is that many of the ions desorb before they reach the state of maximum charge permitted by the surface charge density. Consequently, the peak heights start decreasing rapidly with decreasing *m/z*, finally disappearing altogether at that value of *m/z* for which the residence time is too short for any of the analyte species to acquire all the charges it can hold. It is this argument that was behind our earlier suggestion that kinetic factors might well account for the observation that the number of charges found on PEG ions never reached the maximum predicted by the electrostatic model. They desorb before they reach that maximum charge state.

A provocative feature of the spectra in Figure 5 is that the minimum and maximum values of *m/z* for the desorption window are about the same for both PEGs even though their molecular weights differ by a factor of 2. Indeed, the windows for all PEGs with molecular weights above 1000 are bounded by nearly the same *m/z* values. Large changes in solute concentration, flow rate, and additions of various cosolutes such as KCl and NaOH do not change these upper and lower limits of *m/z*, even though they do affect the shape of the profile in between them. This near equality of the upper and lower *m/z* limits for all the relatively large PEG ions can be understood in terms of their structural similarity.

According to the arguments already set forth, the Na^+ are attached to O atoms and are pretty much uniformly distributed along the oligomer backbone. Thus, the lifting force due to the interaction of the Na^+ and the field at the droplet surface is also rather uniformly distributed along the length of the molecule as is

the solvation force binding the ion to the surface. Clearly, the necessary and sufficient condition for desorption is that the local lifting force exerted on an Na^+ by the field at the droplet surface exceed the local solvation force. The structure of a large oligomer is simply a repetitive sequence of essentially identical units, each comprising a section of chain length associated with an Na^+ that lifts it off when the field is high enough. It follows that the maximum value of *m/z* at lift-off should be pretty much the same for oligomers of any length.

As was pointed out earlier, Coulomb repulsion forces result in an equidistant distribution of charges along the molecule with the first and last charges as near to each end as possible. If we make the reasonable assumption that the mass per unit length is roughly constant, then *m/z* becomes proportional to $L(z - 1)/z$ where L is the distance between charges which we have assumed to be roughly constant for the maximum value of *m/z* at "lift-off". Thus, as m and therefore z increase, *m/z* also increases. Consequently, the maximum *m/z* value for small oligomers should be less than for large ones. The spectra in Figures 4 and 5 confirm this expectation.

The near equality of *minimum m/z* values observed for PEG ions of all larger sizes can also be understood in terms of similar arguments. The value of *m/z* at which the time to desorption for an ion becomes less than the time required for additional charging should, because of local similarity, also be relatively independent of oligomer size. However, for a given average distance between charges, the electrostatic potential energy for a charge on a large oligomer will be higher than for one on a smaller oligomer simply because there are more charges contributing to that energy. That is to say, all the charges on an oligomer contribute to the repulsive potential energy of any one charge. Therefore, to achieve a particular average charge distance will require a higher charge density on the droplet surface for a large oligomer than for a small one. Conversely, at a particular surface charge density the average distance between charges will be smaller on a small oligomer so the minimum *m/z* value it can reach before lift-off will also be smaller. Thus, the lower limit for *m/z* becomes somewhat smaller with decreasing oligomer mass as the spectra in Figures 4 and 5 indicate. The curvature of the solid line in Figure 6 also stems from this same effect. At the point of equality between the energy of 2.05 eV that bonds an Na^+ to an O atom in PEG and the electrostatic potential energy of repulsion, the spacing between charges must be greater on a large oligomer than on a small one simply because there are more charges contributing to that energy on the large oligomer.

As noted earlier, the spectra for proteins in Figure 7 show well-separated peaks for ions of differing charge numbers. The envelopes of these peaks have shapes similar to the profiles of the bands of unresolved peaks in the larger PEGs of Figure 5, defining a "window" of desorption with upper and lower limits to observed values of *m/z*. The existence of these limits can be understood for proteins in terms of the same kinetic arguments that were applied to the PEG case. Proteins, however, do not have the structural uniformity of PEGs nor is their structural similarity as independent of molecular weight. Consequently, one might expect that the experimental values for the upper and lower limits of *m/z* should vary much more for proteins than for PEGs, as indeed they do in the spectra of Figure

7 and Table I. Even so, for all the species thus far investigated, the upper end of the desorption window has almost always been at *m/z* values less than 1500. Only for α-chymotrypsinogen and lysozyme did the envelope of peaks seem to extend beyond this value, perhaps by 100 or 200 units.

Upon reflection, this behavior is perhaps not so surprising. Many proteins are alike in that they all comprise amino acids that are themselves sufficiently similar so that the population density of polar sites for ion attachment is probably not all that different from protein to protein. Moreover, above some size sufficient to retain several charges, one end of a molecule that is "stretched out" by repulsive forces due to the plurality of its charges cannot very well "know" what the other end is doing. In other words, the local desorption forces depend upon the local charge density on the droplet surface and probably reach the lift-off condition at about the same time all along the length of the molecule. Because the number densities and spatial distributions of prospective charge sites vary from protein to protein, the variation in the position and width of the desorption window shown in Table I is to be expected. It may emerge that these features of the ES mass spectra can be a source of information on the identity and/or structure of the parent molecule. In this perspective it is clear that the sometimes-asserted simple proportionality between the number of basic amino acid units in a peptide and the number of charges it retains, is a gross oversimplification.

One inviting consequence of the preceding scenario is that the ultimate upper limit for mass at which any sufficiently polar species can be desorbed as an ion might occur only when that species is long enough to encircle the droplet! If so then many very large molecules of great biochemical moment would come within reach of mass spectrometric examination. Two bits of support for this speculation are presented in Figure 8. Panel (a) shows evidence that biopolymers quite different from peptides and proteins can be successfully ionized by ES. It comprises the negative ion spectrum for an oligonucleotide obtained by Covey et al. (41) using so-called "Ionspray" (IS) that we discussed earlier. (At the sample injection rate of 4 μL/min used for this spectrum the aerodynamic assist, which is supposed to distinguish IS from ES, is not needed and is probably counterproductive. Indeed, all the reported IS results showing capability of high mass and/or high sensitivity have been obtained with liquid flow rates well within the acceptable range for pure ES.)

Figure 8(b) shows an ES spectrum obtained by Smith et al. (42) for the dimer of bovine albumin having a molecular weight of 133,000. The number of charges/ion seems to range from about 80 to 130 and the desorption window lies between *m/z* values of 900 and 1700. When *m* and *z* are so large the peaks may be more difficult to resolve because the difference in *m/z* values for adjacent peaks is very small. Evidence of this kind of resolution problem is also apparent in our spectrum for alcohol dehydrogenase shown in Figure 7. Though to generalize from a limited experimental base is dangerous, it would appear from the results to date that the window of desorbability will seldom if ever have to be open above an *m/z* value of 3000 or so, well within the reach of a good quadrupole mass filter.

A significant implication of these observations is that to exploit the advantages of ES ionization, the appeal of an analyzer will depend more upon its resolving

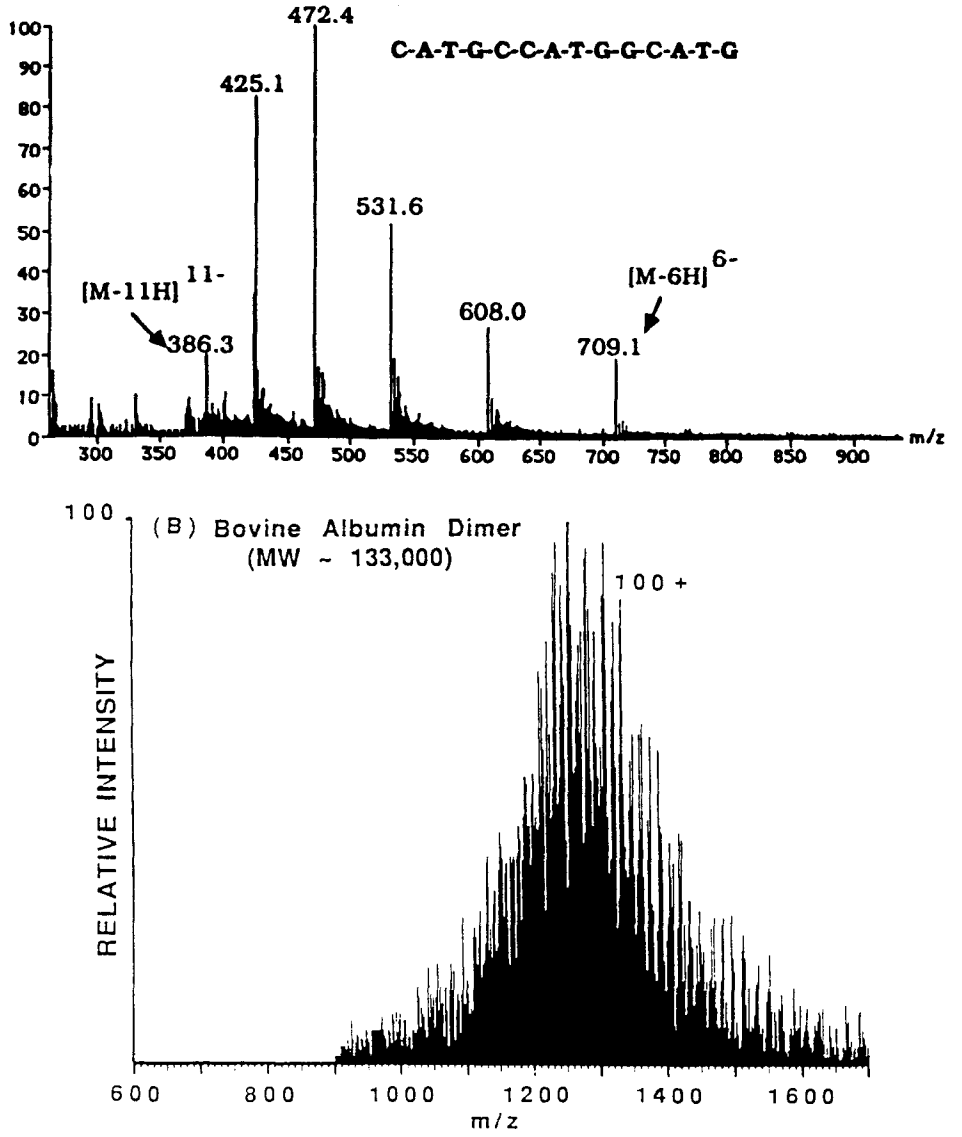

Figure 8. Electrospray mass spectra for large molecules by other investigators: (a) for a synthetic oligonucleotide (Ref. 42). (b) For a larger protein (Ref. 43).

power than its ability to accommodate ions with high values of *m/z*. In this perspective Fourier transform ion cyclotron resonance (FTICR) instruments would seem to be the analyzers of choice. Up to now such analyzers have for several reasons had less than spectacular success with ions of high mass. Their low orbit frequencies require long integrating times and cannot be measured with the precision obtainable at high frequencies. In turn, long integrating times require operation in ultrahigh vacuum in order to avoid orbit perturbation by collisions with

background gas molecules. We think that the extensive multiple charging characteristic of ES ions may reduce their m/z values sufficiently to avoid these problems of low frequency operation. In addition, the large number of charges should induce much larger currents per ion in the external detection circuit. Another possible bonus: because of multiple charges ES ions of very high m will still have high velocities. The associated momentum will be so large that orbit perturbation due to collisions with background gas molecules should be minimal. Consequently, acceptable operation may not require ultra high vacuum. Of course, to realize these prospective advantages one must be able to transfer ions from an external ES source into an ICR cavity. Investigators are pursuing techniques for such transfer and have already achieved modest success with other kinds of sources. It is our guess that ES sources should be equally tractable.

D. Interpreting the spectra of multiply charged ions

The instinctive response to results of the kind shown in Figures 7 and 8 is one of horror by mass spectrometrists whose experience has been almost entirely concerned with singly charged ions. The presence in a spectrum of several different parent peaks for a single species seems to promise great difficulties in interpretation. In fact, the multiplicity of peaks in an ES spectrum turns out to offer some substantial advantages. Clearly, the m/z value for each such peak has three unknown components, the mass of the parent molecule, the number of charges on the ion, and the mass of each attached species contributing one of those charges. One would expect a priori that the ions of adjacent peaks should differ by one charge because it is hard to imagine a physical reason why a parent molecule should collect charges two or more at a time rather than one at a time. This expectation has been born out a posteriori by all experiments to date. Therefore we can safely assume that the peaks in sequences of the kind shown in Figures 7 and 8 are "quantized" in the sense that the ions of adjacent peaks always differ by one charge.

This "coherence" of peaks in an ES spectrum allows one to identify and eliminate from consideration those peaks that do not belong in a sequence. It also reduces to two the number of independent variables per peak so that measured m/z values for any two peaks in the sequence are sufficient to specify the masses of both the parent ion and the charge bearing species. Clearly, if the latter differ from peak to peak, more information will be needed, but all experiments indicate that in many, if not most, cases the charge-bearing species is the same for all peaks in a sequence. The net result is that each peak becomes in effect an independent measure of the parent ion mass. Therefore, one can signal-average over the peaks in a single spectrum to reduce random error and increase both the precision of and confidence in the mass assignment.

We have developed two algorithms that allow one to take advantage of the information provided by peak multiplicity (43). The "signal-averaging algorithm" displays mass values obtained from the m/z values of all possible pairs of peaks, identifies peaks that do not belong in the sequence, indicates the overall quality of the spectrum, and provides the basis for obtaining a weighted average for the

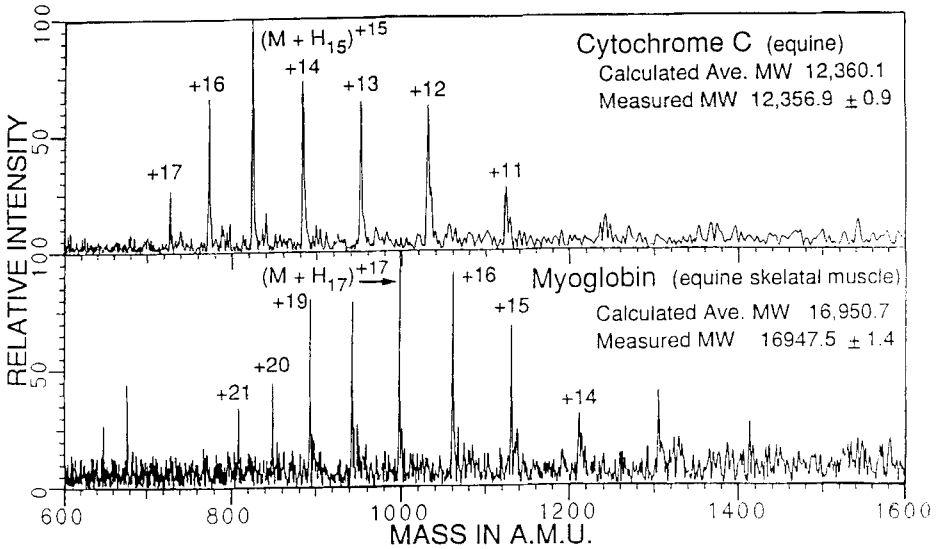

Figure 9. Electrospray mass spectra obtained by coupling an ES source to a Fourier transform mass spectrometer (Ref. 44).

parent mass. The "deconvolution algorithm" instructs the computer to scan the spectrum, adding contributions from each peak that fits in the sequence, and presents the results as a single peak with an m/z value corresponding to the parent molecule with one massless charge attached. Of course, the accuracy and precision with which mass assignments can be made depend ultimately upon the accuracy and resolution of the analyzer's m/z scale. The contribution of peak multiplicity is to reduce random error in the location of any one peak by about $\sqrt{n}$ when n is the number of spectral peaks over which signal averaging takes place. Thus, the random error in the average value of m/z for a spectrum with 10 peaks is less than 1/3 of the uncertainty in locating any single peak. Some of our preliminary experiments have shown that these algorithms can resolve the components of mixtures even when the peaks in the raw spectrum seem hopelessly "incoherent" to the naked eye.

V. SUMMARY AND CONCLUSIONS

This article has attempted to provide a perspective on electrospray ionization by reviewing its achievements and speculating on its prospects. The important features that ES seems to offer practicing mass spectrometrists include: (1) minimal fragmentation of parent species, no matter how large or fragile; (2) remarkable sensitivity in terms both of analyte concentration and sample size; (3) intact ions from any solute species containing polar atoms or groups, even when the parent

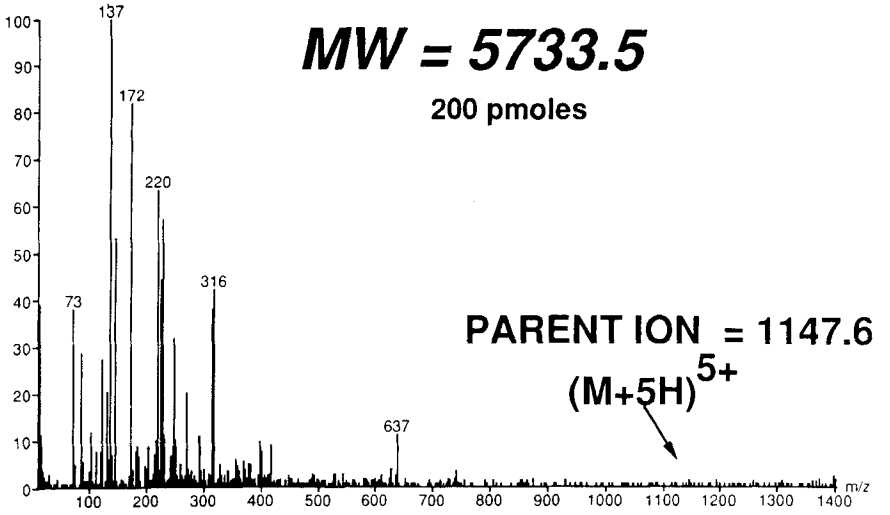

Figure 10. MS/MS of the bovine insulin parent molecule with five charges (Ref. 45).

has a six-figure molecular weight; (4) multiple charging so extensive that ion *m/z* values are always less than about 2500, no matter how massive the parent species; (5) spectral peak multiplicity that can substantially reduce random error in mass assignment.

Our aim has been to present a balanced view, but we admit that our own enthusiasm may have led to occasional hyperbole. We are hopeful and confident that our fellow citizens in the community of mass spectrometry will ultimately show that what may now seem exaggeration will, in fact, turn out to be understatement.

VI. ACKNOWLEDGMENTS

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VII. EPILOG

Since this manuscript was completed and submitted for review results have been emerging at a rapidly increasing rate from a number of laboratories that have been attracted by the promise of ES mass spectrometry. This heightened interest and activity was clearly evident at the annual meeting of the American Society for Mass Spectrometry at Miami in May 1989. Of the many ES results reported there, we identify two revelations that seem particularly important. They also happen to relate to some of our speculation in this report. The first one makes us look prescient. The second confirms our fallibility!

1. F.W. McLafferty presented the exciting results shown in Figure 9, obtained in a collaboration between his group at Cornell and D.F. Hunt's at Virginia (44). The ions were obtained with an external ES source and were transferred through a quadrupole "conduit" into the cell of an FTICR mass spectrometer. These landmark spectra are for the largest ions by far that have ever been analyzed by this technique. They constitute welcome confirmation of our speculation that the marriage of ES and FTICR should be a fruitful union.

2. Figure 10 displays the spectrum of daughter ions obtained by fragmenting multiply charged ions of bovine insulin (45). We have space here only for this sample of the preliminary but most promising results presented by T. Covey and his colleagues on MS-MS of multiply charged ions from peptides and proteins. Analogous but less extensive experiments were also reported by R.D. Smith and his collaborators. The exciting message is that multiply charged ions of the kind produced by ES are readily fragmented by collisions at relatively low energy with argon atoms. It turns out that the higher the charge multiplicity, the lower is the required energy. Earlier in this account we expressed concern about possible difficulties in getting enough collision energy in the center of mass to fragment large ions by collision with gas-phase target molecules. These apprehensions were clearly without foundation. We failed to take into sufficient account the contribution of multiple charging to effective collision energy as well as to the stress that the resulting Coulomb repulsion seems to place on the intramolecular bonds. We are delighted to have been wrong!

REFERENCES

1. (a) Dole, M.; Mach, L. L.; Hines, R. L.; Mobley, R. C.; Ferguson, L. P.; Alice, M. B. *J. Chem. Phys.* **1968,** *49,* 2240; (b) Mach, L. L.; Kralik, P; Rheude, A.; Dole, M. *J. Chem. Phys.* **1970,** *52,* 4977; (c) Clegg, G. A.; Dole, M. *Biopolymers* **1971,** *10,* 821; (d) Teer, D.; Dole, M. *J. Polym. Sci.* **1975,** *13,* 985.
2. Beuhler, R. J.; Flanigan, E; Green, L. J.; Friedman, L. *J. Am. Chem. Soc.* **1974,** *96,* 3990.
3. (a) Meuzelaar, H. L. C.; Posthumus, M. A.; Kistemaker, P. G.; Kistemaker, J. *Anal. Chem.* **1973,** *45,* 1546; (b) Kistemaker, P. G.; Boerboom, A. J. H.; Meuzelaar, H. L. C. *Dyn. Mass. Spectr.* **1976,** *4,* 139.
4. (a) Unsöld, E.; Hillenkamp, F.; Nitsche, R. *Analysis* **1976,** *4,* 115; (b) Posthumus, M. A., Kistemaker, P. G.; Meuse, Ten Noever de Brauw, M. C. *Anal. Chem.* **1978,** *50,* 985.
5. Devienne, F. M.; Giroud, J. 5th Int'l Symp. Mol Beams (Abstr B-7) Nice 1975; cf Devienne, F. M.; Roustan, J. C. *Org. Mass Spectrom* **1982,** *17,* 1973.
6. Barber, M.; Bordoli, R. S.; Elliot, G. J.; Sedgwick, R. D.; Tyler, A. N. *J. Chem. Soc. Chem. Commun.* **1981,** 325.

7. (a) Stroh, J. G.; Cook, J. C.; Milberg, R. M.; Brayton, L.; Kihara, T.; Huang, Z.; Rhinehart *Anal Chem.* **1985,** *57,* 985; (b) Dobberstein, P.; Karte, E.; Meyerhoff, G.; Pesch, R. *Int. J. Mass Spectrom. Ion Phys.* **1983,** *46,* 185.
8. (a) Caprioli, R. M.; Fan, T.; Cottrell, J. S. *Anal. Chem.* **1986,** *58,* 2949; (b) Caprioli, R. M. *Mass Spectrom. Rev.* **1987,** *6,* 237.
9. Benninghoven, A.; Jaspers, D.; Sichtermann W. *Appl. Phys.* **1976,** 11, 35.
10. Honig, R. E. *Int. J. Mass Spectrom. Ion Phys.* **1985,** *66,* 31.
11. (a) Torgerson, D. F.; Skowronski, R. P.; Macfarlane, R. D. *Biochem. Biophys. Res. Commun.* **1974,** *60,* 616; (b) Sundqvist, B. U. R.; Macfarlane, R. D. *Mass Spectrom. Revs.* **1985,** *42,* 421; (c) Cotter, R. *Anal. Chem.* **1988,** *60,* 781A.
12. (a) Karas, M.; Killenkamp, F. *Anal. Chem.* **1988,** *60,* 2299; (b) Karas, M.; Hillenkamp, F. Paper presented at the 11th International Mass Spectrometry Conference, Bordeaux, France, 1988 and ASMS Workshop on Ion Desorption Mechanisms, Sanibel Island, Florida, January 1989.
13. Barber, M.; Green, B. N. *Rapid Commun. Mass Spectrom.* **1987,** *1,* 80.
14. Jonsson, G.; Hedin, A.; Hakansson, P.; Sundquist, B. U. R.; Bennich, H.; Roepstorff, P. *Rapid Commun. Mass Spectrom.* **1989,** *3,* 190.
15. Russell, D. H. *Mass Spectrom. Revs.* **1986,** *5,* 167.
16. Schey, K.; Cooks, R. G.; Grix, R.; Wollnik, H. *Int. J. Mass Spectrom. Ion. Proc.* **1987,** *77,* 49.
17. (a) Müller, E. W. *Z. Physik* **1951,** *131,* 136; (b) "Electronic and Electron Physics"; Vol. XIII, pp. 83–79 Academic Press. New York, 1960.
18. Inghram, M.; Gomer, R. J. *J. Chem. Phys.* **1954,** *22,* 1279.
19. Beckey, H. D. "Principles of Field Ionization and Field Desorption Mass Spectrometry."; Pergamon Press: Oxford, 1977.
20. Evans, C. A. Jr; Hendricks, C. D. *Rev. Sci. Instr.* **1972,** *43,* 1527.
21. (a) Simons, D. S.; Colby, B. N.; Evans, C. A. Jr. *Int. J. Mass Spectrom. Ion Phys.* **1974,** *15,* 291; (b) Cook, K. D. *Mass Spec. Revs.*, **1986,** *5,* 467.
22. Iribarne, J. V.; Thomson, B. A. *J. Chem. Phys.* **1976,** *64,* 2287.
23. Chapman, S. *Phys. Rev.* **1937,** *52,* 184; ibid **1938,** *54,* 520, 528.
24. (a) Thomson, B. A.; Iribarne, J. V. *J. Chem. Phys.* **1979,** *71,* 4451; (b) Iribarne, J. B.; Dziedzic, P. J.; Thomson, B. A. *Int. J. Mass Spectrom. Ion Phys.* **1983,** *50,* 331.
25. (a) Blakley, C. R.; McAdams, M. J.; Vestal, M. L. *J. Chromatogr.* **1978,** *158,* 264; (b) Blakley, C. R.; Carmody, J. J.; Vestal, M. L. *Anal. Chem.* **1980,** *52,* 1636; (c) Blakley, C. R.; Carmody, J. J.; Vestal, M. L. *Clin. Chem.* **1980,** *26,* 1467; (d) Blakley, C. R.; Carmody, J. J.; Vestal, M. L., *J. Am. Chem. Soc.* **1980,** *102,* 5931; (e) Vestal, M. L. in "Mass Spectrometry in the Health and Life Sciences"; Burlingame, A. L.; Castagnoli, N., Jr., Eds, Elsevier: Amsterdam, **1985,** pp. 99–118.
26. Whitehouse, C. M.; Dreyer, R. N.; Yamashita, M.; Fenn, J. B. *Anal. Chem.* **1985,** *57,* 675.
27. (a) Yamashita, M.; Fenn, J. B. *J. Phys. Chem.* **1984,** *88,* 4451; (b) Yamashita, M.; Fenn, J. B. *J. Phys. Chem.* **1984,** *88,* 4471.
28. Whitehouse, C. M.; Yamashita, M.; Meng, C. K.; Fenn, J. B., "Proceedings of the 14th International Symposium on Rarefied Gas Dynamics, July 16-20, **1984**"; Oguchi, H., Ed., University of Tokyo Press, Tokyo, 1984, p. 857.
29. (a) Doyle, A.; Moffett, D. R.; Vonnegut, B. *J. Colloid Sci.* **1964,** *19,* 136; (b) Schweitzer, J. W.; Hanson, D. N. *J. Colloid Sci.* **1971,** *35,* 417; (c) Abbas, M. A.; Latham, J. *J. Colloid Mech.* **1967,** *30,* 663.
30. Abuaf, N.; Anderson, J. B.; Andres, R. P.; Fenn, J. B.; Miller, D. R. In "Rarefied Gas Dynamics"; C. L. Brundin, Ed., Academic Press: New York, **1967,** v. 2, p. 1317; Anderson, J. B. *Entropie* **1976,** *18,* 33.
31. (a) Röllgen, F. W., Bramer-Weger, E.; Bütfering, L. *J. de Physique* **1987,** *48,* C6-253. (b) Schmelzeisen-Redeker, G.; Bütfering, L.; Röllgen, F. W. *Int. J. Mass Spectrom. Ion Proc.* **1989,** *90,* 139.
32. Beuhler, R. J.; Friedman, L.; *Nucl. Inst. Met.* **1980,** *170,* 309.
33. (a) Olivares, J. A.; Nguyen. N. T.; Yonker, C. L. R.; Smith, R. D. *Anal. Chem.* **1987,** *59,* 1230; (b) Smith, R. D.; Olivares, J. A.; Nguyen, N. F.; Udseth, H. R. *Anal. Chem.* **1988,** *60,* 436.

34. Smith, R. D.; Barinaga, C. J.; Udseth, H. R. *Anal. Chem.* **1988,** *60,* 1948.
35. (a) Bruins, A. P., Covey, T. R., Henion, J. D., *Anal Chem.* **1987,** *59,* 2642; (b) Bruins, A. P.; Weidolf, L. O. G. Henion, J. D. *Anal. Chem.* **1987,** *59, 2647.*
36. Henion, J. D. Results presented at ASMS Workshop on Desorption Mechanisms, Sanibel Island, Florida, January **1989.**
37. Wong, S. F.; Meng, C. K.; Fenn, J. B. *J. Phys. Chem.* **1988,** *92,* 546.
38. (a) Biemann, K. *Anal. Chem.* **1986,** *58,* 1288A; (b) Biemann, K.; Martin, S. A. *Mass Spectrom. Rev.* **1987,** *6,* 1.
39. (a) Meng, C. K.; Mann, M.; Fenn, J. B. *Zeit. Phys. D* **1988,** *10,* 361; (b) Mann, M.; Meng, C. K.; Fenn, J. B. In Proceedings of the 36th Annual Conference on Mass Spectrometry and Allied Topics, San Francisco CA, June **1988,** pp. 1207–8.
40. Mann, M. "Quantitative Aspects of Electrospray Mass Spectrometry," Ph.D. thesis, Yale University, University Microfilms: Ann Arbor, 1989.
41. Covey, T. T.; Bonner, R. F.; Shushan, B. I.; Henion, J. D. *Rapid Commun. Mass Spectrom.* **1988,** *2,* 249.
42. Smith, R. D.; Loo, J. A.; Barinaga, C. J.; Udseth, H. Presentation at 5th (Montreux) Symposium on LC-MS, Freiburg, November *1988.*
43. (a) Mann, M.; Meng, C. K.; Fenn, J. B. *Anal. Chem.* **1989,** *61,* 1702; (b) Mann, M.; Meng, C. K.; Fenn, J. B. In Proceedings of the 36th Annual Conference on Mass Spectrometry and Allied Topics, San Francisco CA, June **1988.**
44. McLaffery, F. W. et al. Presented at the 37th Annual Conference on Mass Spectrometry and Allied Topics, Miami FL, May **1989.**
45. Covey, T. R. et al. Presented at the 37th Annual Conference on Mass Spectrometry and Allied Topics, Miami FL, May **1989.**