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Of protons or proteins

"A beam's a beam for a' that." (O.S. Burns)

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Mass analyses have been carried out on ions produced by an Electrospray (ES) source from dilute solutions of protein molecules with molecular weights (M) in the range from 5000 to nearly 40000. Each spectrum comprises a sequence of peaks corresponding to multiply charged intact parent species. The ions of each peak differ from those of their adjacent neighbors by one unit charge, H^+ in these experiments. The maximum number of charges per ion generally increases with the molecular weight of the parent molecule, reaching a value of 45 in the case of alcohol dehydrogenase, at $M = 39830$ the largest species in this study. Thus the resulting values of m/z are within reach of a simple quadrupole mass filter whose nominal upper mass limit is 1500 daltons! The immediate application for the ES source is in mass spectrometric analysis of large fragile molecules of biochemical importance. But the multiply charged ions it produces are newcomers to the laboratory scene that constitute interesting subjects for study.

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I. Introduction

For some time we have been investigating Electrospray (ES) ionization, a "soft" method of producing intact ions in vacuo from fragile and non volatile species in solution so that they can be analyzed by mass spectrometry. This method has its operational roots in two techniques: *(i)* the dispersion of liquids into charged droplets by strong electric fields and *(ii)* the transport of molecular species from the hustle and bustle of gaseous chaos into splendid isolation as ordered beams in vacuum. It was Otto Stern who first recognized what could be done with such beams and introduced them to the world of science as a powerful research tool. In our experiments, for reasons that will emerge, it is convenient and advantageous to provide this transport into vacuum by the supersonic free jets that have become a common and fruitful alternative to the effusive sources originated by Stern.

It seems historically appropriate to note that in the form we use them these two techniques were first reduced to effective practice in the same institution that is now home for our laboratory. The first serious experimental study of electrostatic dispersion of liquids was by Zeleny at Yale in 1917, just about the time that Stern was preparing to carry out his first beam experiments at Frankfurt [1]. Moreover, though unappreciated at the time, and often not realized since, the first use of supersonic free jets as sources for molecular beams was also at Yale – by Johnson in 1927, near the midpoint of Stern's stay in Hamburg [2].

The first use of ES ionization for Mass Spectrometry was in the pioneering experiments of Malcolm Dole and his colleagues two decades ago. They attempted, with some apparent success, to produce beams of macroions, first from polystyrene molecules and later from zein and polyvinylpyrrolidone [3]. However, their interpretation of what they observed hinged on the assumption of velocity equality between carrier gas and macroions during free jet expansion into the vacuum system. This assumption and, therefore, the conclusions it led to, are open to question. We repeated Dole's experiments not long after they were published and confirmed his observations. Then he and we both abandoned further efforts

because the primary ion currents were very small and could not be enhanced by the millionfold gain commonly achieved with ion multiplier detectors. Such large ions would not produce secondary electrons by colliding with a surface unless, as was later learned, they were first accelerated to half a million volts or so [4]. Moreover, the mass analyzers then available were not effective in the desired mass range (tens of kilodaltons). About five years ago we took another look at the ES ionization process, applying it to smaller ions for which available mass analyzers and multiplier detectors performed well. It has turned out to be a much more interesting phenomenon and powerful technique than we had first thought [5].

II. Apparatus and method

Our particular combination of techniques has been previously described, but for the convenience of readers who may not be familiar with how they are embodied in an ES mass spectrometer we begin by describing the apparatus with which we obtained the results to be presented. The schematic representation in Fig. 1 together with the following description of its operation provides a convenient introduction to its principles [6]. 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. Typical values of applied voltages are in the 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 the quadrupole

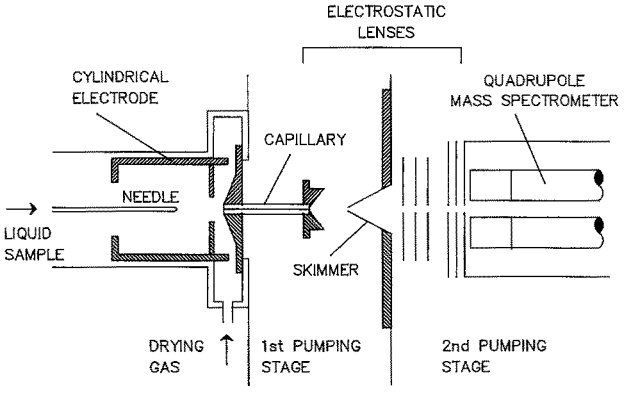

Fig. 1. Schematic diagram of the apparatus for mass spectrometry with an electrospray ion source

(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 in order to inhibit the onset of a corona discharge which occurs at lower voltages in the negative ion mode. The indicated potential difference of 4540 V between inlet and exit ends of the capillary may seem startling. We have found that with the bath-carrier gas (nitrogen) at about 1 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 to ground or to as much as 15 kV above ground potential. 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 (0.1 mm in diameter) that it replaced. With this configuration all external parts of the apparatus are at ground and pose no hazard to the operator.

The field at the needle tip charges the surface of the emerging liquid which then becomes dispersed 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 counter-current flow of bath gas typically at 800 torr and an entering temperature from 320–350 K at a flow rate of about 100 ml/s. As they rapidly evaporate en route, the resulting sovent vapor along with other uncharged material is swept away from the capillary inlet by the bath gas flow. Meanwhile, the rapid evaporation of a migrating droplet increases the charge density on its surface until the Rayleigh limit is reached at which the Coulomb repulsion approaches the surface tension and the droplet finally "explodes". Each of the resulting daughter droplets is also charged and continues to evaporate until the Rayleigh limit is again approached and another Coulomb explosion occurs. This sequence repeats until finally the radius of curvature of the droplet is so small that the electric field at its surface is high enough to desorb solute ions from the droplet liquid into the ambient gas. Even solute species that are not themselves 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.

This explanation of ion formation, first proposed by Iribarne and Thomson, seems to be the underlying mechanism not only for ES but also for the Aerospray (AS) source of Thomson and Iribarne, (called by them the Atmospheric Pressure Ion Evaporation (APIE) source) and the widely used Thermospray (TS) source of Vestal and his colleagues [7, 8]. We should point out that according to the mechanism originally as-

sumed by Dole, the ultimate droplet in the sequence of Coulomb explosions would contain only one molecule of solute if the original solution were sufficiently dilute. As the last bit of solvent evaporated the remaining solute molecule would retain the droplet's charge, thus becoming an ion. The ion desorption model of Iribarne is now more generally accepted but under some conditions the Dole model may apply and probably did in his experiments with polystyrene which have no polar groups and, therefore, cannot readily become charged by attaching solute ions. However, there is reason to believe that Dole's ultimate droplets contained more than one molecule and more than one charge. Thus, some of his ions were probably multicharged clusters whose combination of m/z and velocity gave them an energy that would make his curves for current-vs-retarding-potential look like those for singly charged molecules at the jet gas velocity.

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 the charging is brought about by atomizing an ion-containing liquid, statistical fluctuations in the distribution of cations and amions among the droplets accounting for their charge. In ES the 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 differences in performance that are important in analytical applications. AS and TS work best with flow rates in the 0.5 to 2 ml/min range encountered in conventional liquid chromatography (LC). ES prefers flow rates below 40 µl/min and thus can work with smaller amounts of analyte. Such low flow rates seem better matched to LC on the microbore scale which is growing in popularity. They are also 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. An ES mass spectrometer seems to be an exceedingly promising detector for CZE applications [9]. ES dispersion does not yet work well with liquids having electrical conductivities higher than those of 0.005 molar KCl solutions. With volatile buffers AS and TS encounter no such difficulties until much higher concentrations are reached. 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. Where they have been compared, ES has shown somewhat greater sensitivity and can accommodate to smaller samples than can AS and TS. We have achieved sensitivities in the attomole range with peptides having masses in the kilodalton range.

A most exciting and attractive feature of ES has been its recently demonstrated ability to produce ions from much larger solute species than either AS or TS and with much higher efficiency than other "soft" ionization techniques. Moreover, it has shown a remarkable ability to achieve much more extensive multiple charging than any other source. A recent paper reported results obtained by ES ionization of polyethylene glycol samples with nominal molecular weights from 200–17500 [10]. The term "nominal molecular weight" refers to the most abundant oligomer in a mixture containing a rather broad distribution of sizes. We found that the number of charges per oligomer ion increased steadily with increasing molecular weight, reaching an average value of 23 for oligomers with a range of molecular weights between 15000 and 20000, i.e. in a sample with a nominal molecular weight of 17500. Thus we could readily analyze ions with masses of 20 kilodaltons even though the nominal upper limit of our quadrupole mass filter was only 1500 daltons.

Each of these PEG samples comprised oligomers with a broad range of molecular weights and the number of charges per ion could vary substantially, especially for the larger oligomers. The resulting number of permutations and combinations of charge and mass was so large that within a unit interval in m/z there were as many as 5 or 6 ions with different masses. Under these circumstances our analyzer could not resolve individual peaks so that the mass spectra for these material comprised the envelope of a broad band of unresolved peaks having a Gaussian-like distribution. Such congestion would be avoided by the use of sample 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. An even 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 [11]. Consequently, we undertook the experiments whose results are reported here.

III. Results and discussion

Most of the samples used in this study came from Sigma Chemical but some were given to us by other laboratories on the campus and had a somewhat uncertain pedigree. We found that the most effective solvents were mixtures of acetonitrile, water and metha-

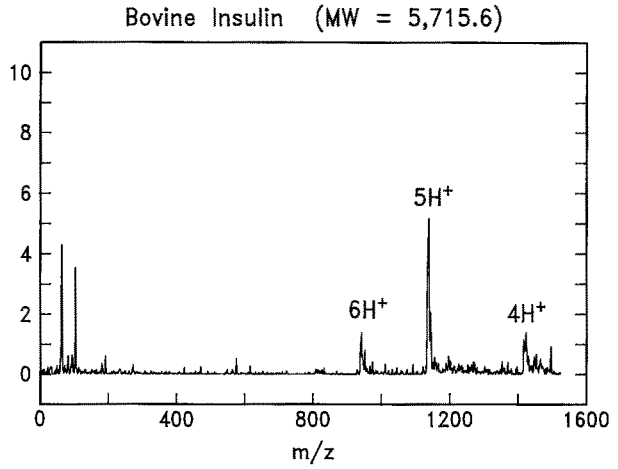

Fig. 2. Electrospray mass spectrum of Insulin from Bovine Pancreas ($M = 5715.6$). Single 30 s scan with 0.05 mg/ml in 45:45:10 MeOH:ACN:H_2O + 0.1% HAc injected at 8 µl/min

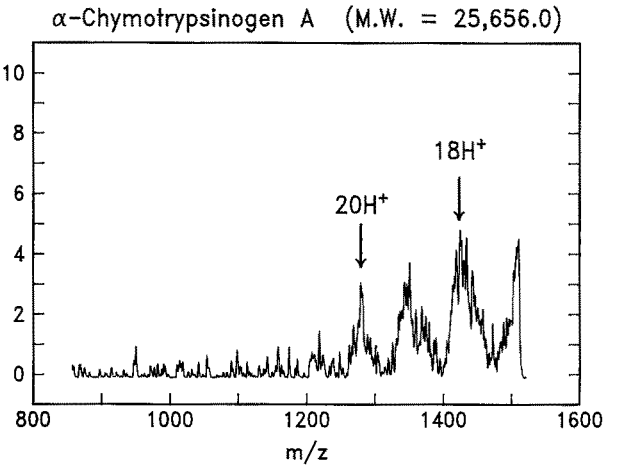

Fig. 4. Electrospray mass spectrum of α-Chymotrypsinogen *A* from Bovine Pancreas ($M = 25656.0$). Single 30 s scan with 0.5 mg/ml in 45:45:10 MeOH:ACN:H_2O + 0.1% HAc injected at 8 µl/min

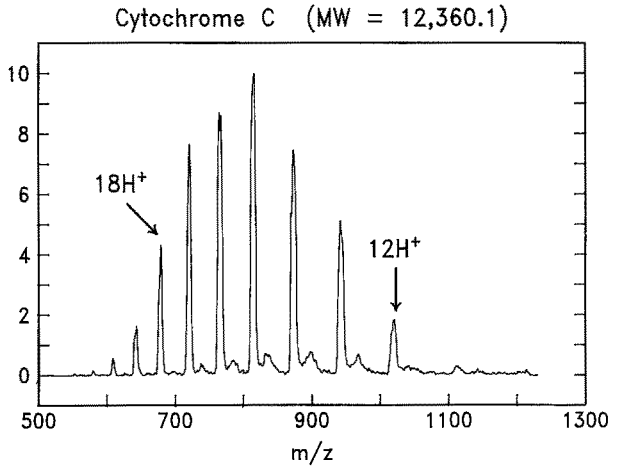

Fig. 3. Electrospray mass spectrum of Cytochrome *C* from Horse Heart ($M = 12360.1$). Single 30 s scan with 1.67mg/ml in 45:45:10 MeOH:ACN:H_2O + 167 ppm TFA injected at 8 µl/min

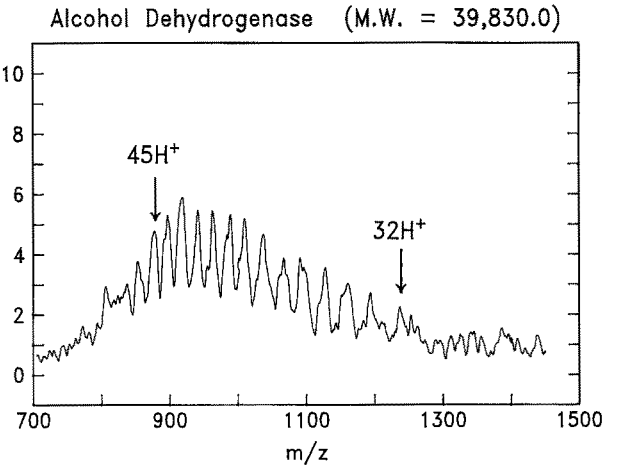

Fig. 5. Electrospray mass spectrum of Alcohol Dehydrogenase from Equine Liver ($M = 39830.0$). Single 30 s scan with 0.5 mg/ml in 1:1 1-propanol:H_2O + 50 ppm TFA injected at 8 µl/min

nol or 1-propanol. It was necessary to lower the solution pH by addition of small quantities of acetic and (HAc) or trifluoroacetic acid (TFA). The optimum proportions of these solvent components depend somewhat on the particular sample and were determined by trial and error. Solutions with analyte concentrations ranging from 0.7–137 µmols/L, depending upon the species, were injected at flow rates of 8 µl/min. All the spectra were obtained by means of a single scan requiring 30 s to cover the indicated mass range. The analog output from the Channeltron detector was digitized with an *A* to *D* converter and fed into a homemade data processing and recording system based on a PC AT clone.

Results are presented for eight proteins whose molecular weights spanned the range between 5000 and 40000. Figures 2–5 show representative mass spectra obtained respectively with Bovine Insulin ($M = 5715.6$), Cytochrome *C* ($M = 12360.1$), α-Chymotrypsinogen *A* (25656.0) and Alcohol Dehydrogenase ($M = 39830.0$). In each case the spectrum comprises a sequence of peaks each of which differs from its neighbor by one charge. As a frame of reference, the number of charges per ion is shown for two or three peaks in each spectrum. This charge due to adduct ions is expressed in terms of the number of protons. In fact, our quadrupole analyzer does not have sufficient resolution for large ions at these m/z values to permit an unequivocal assertion that each adduct charge has unit mass and is a therefore a proton. However, the need for low pH in the sample solution along with results obtained for smaller peptides and amino acids strongly support the assumption that H^+ is the most likely charge carrier.

Table 1

	Concentration		Charges		Molecular weight solute	
	Gms/L.	μMol/L.	per ion	*m/z* Range	actual	measured
Bovine Insulin	0.05	8.8	4– 6	950– 1450	5715.6	5720
Cytochrome C	1.67	137.0	12–20	600– 1100	12360.1	12322
Lysozyme	0.01	0.71	10–15	900–>1500	14306.1	14272
Myoglobin	1.00	58.8	15–27	600– 1200	16950.0	16966
Trypsin Inhibitor	0.10	5.0	16–22	800– 1400	20090.7	20072
α-Chymotrypsinogen *A*	0.50	19.0	17–21	1150–>1500	25656.0	25768
Carbonic Anhydrase II	0.50	17.2	23–36	725– 1500	29006.0	29000
Alcohol Dehydrogenase	0.50	12.5	32–46	800– 1300	39830.0	39850

For all eight proteins that we studied Table 1 summarizes essential features of their spectra and the information they provided. 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 other "soft" ionization method. This feature is very attractive because the effective mass range of any analyzer is increased by a factor equal to the number of charges per ion. Thus in these experiments we were able to "weigh" ions with masses up to nearly 40 kilodaltons even though the nominal upper limit for our analyzer is only 1500 daltons. 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. This trend is in keeping with our earlier findings with polyethylene glycols (PEGs). In that case oligomers of all sizes had essentially the same structure and chemical composition. We were therefore able to calculate the maximum number of charges that an oligomer of particular size could hold in terms of a model that assumed the charge bearing species comprised cations from the solution (Na^+ in the PEG experiments), were bound to polar sites (O atoms), and were distributed with uniform spacing along the length of the molecule. The model further assumed that the maximum number of charges was reached when the energy with which an Na^+ was bound to an O atom was just equal to the electrostatic repulsive potential energy of the last arriving (centermost) ion calculated as a pairwise sum over all the other charges on the oligomer. It turned out that the number of charges experimentally observed was only about 60 per cent of the maximum number permitted by the model. We are not yet able to carry out a similar calculation for these protein biopolymers because we do not have enough information on their much more complex geometric configurations nor on the binding energies of ions to each of their several kinds of polar sites.

The envelopes of the peaks in all these protein spectra had Gaussian shapes similar to those observed previously in the PEG studies. Then, however, the peaks were unresolved so that the actual charge distribution for a given oligomer could not be distinguished. In the present study we can see clearly that for a given parent species there is a "window" of *m/z* values with a fairly well defined lower limit to which the approach is rather steep in terms of decreasing peak height. The boundary for the upper limit is somewhat less distinct and is approached at a more gradual rate of decrease in peak height. In reflecting on this provocative behaviour we speculate that the *lower* limit on *m/z* (i.e. the maximum number of charges/ion) may be due to the rapid increase in rate of desorption with increasing ionic charge. Thus, an ion may not stay on the surface long enough to acquire all the charges it is capable of retaining. Another possibility is that as evaporation increases its surface charge density a droplet may reach the Rayleigh limit and explode before the charge density is high enough to provide a solute species with all the charge it can hold. Either of these possibilities could account for our previous observation that the number of charges found on PEG ions never reached the maximum predicted by the model.

The *upper* limit on *m/z* can be attributed to the requirement for a minimum number of charges to give the surface field enough leverage to overcome the solvation forces between the solute species and the droplet. In other words, the chemical potential difference between an ion on the surface and above the surface decreases as the surface charge density increases because both the surface field and the number of charges per ion also increase with increasing surface charge. The minimum number of charges that we can observe on a desorbed ion probably corresponds to the density of surface charge at which that difference in chemical potential vanishes.

Also provocative is the fact that with all the species we have thus far investigated the value for m/z at the upper end of the window has almost always been 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 we find this behaviour intuitively reasonable. Proteins are alike in that they all comprise amino acids which 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 the molecule cannot very well "know" what the other end is doing. In other words the local desorption forces depend upon local charge density on the droplet surface and probably reach the "lift-off" value at about the same time all along the molecule. Of course, for species with different number densities and spatial distributions of prospective charge sites the position and width of the window will vary. In the case of all PEGs with molecular weights above 1400 the window extended from m/z values of about 500 to about 1300. For the proteins of this study, as the table shows, the position and width of the desorption window varied substantially from species to species. It may emerge that these features of the ES mass spectrum can be a source of information on the identity and/or structure of the parent molecule.

One inviting consequence of the scenario just outlined is that the ultimate upper limit for mass at which any sufficiently polar solute species can be desorbed as an ion might occur only when the dimensions of that species approach the diameter of the droplet! If so, then many very large molecules of great biochemical moment would be within reach of mass spectrometric probing. Though it is dangerous to generalize from our still limited experimental base we would guess that the window of desorbability will seldom if ever have to be open above m/z of 4000 or so. Consequently, for mass spectrometry of large biomolecules the appeal of an analyzer will depend more upon its resolution than on its ability to accommodate ions with high values of m/z. Indeed, if solutions can be found to the problem of depositing ions from an ES source into an Ion Cyclotron Resonance (ICR) cell, ICR-Fourier Transform mass analysis might well become the technique of choice, especially if high temperature superconductors bring down the costs of producing intense magnetic fields.

To mass spectrometrists accustomed to dealing solely with singly charged ions the prospect of several peaks for each species seems to promise complications in the interpretation of mass spectra containing them. Fortunately, all our experience thus far indicates that ES ionization produces only parent peaks, no fragment ions. (For the few exceptions we have found to this generalization the apparent fragmentation could be attributed to decomposition in the solution, e.g. by hydrolysis, before desorption of the ion from its droplet.) Therefore, if MS analysis is preceded by a separation step, e.g. Liquid Chromatography (LC), peak multiplicity would offer no interpretation problems. Indeed what at first seems to be a vice turns out to be a virtue. Clearly there are three variable unknowns associated with each peak: *(i)* the actual mass of the parent solute species, *(ii)* the number of charges on the ion, and *(iii)* the mass of the adduct carrying the charge. If the mass of this adduct charge is the same for all peaks in a spectrum and there are at least three peaks for which the m/z value is known (i.e. three equations) then one can solve for the value of each of the three variables. Indeed, because the peaks are "quantized" in the sense that adjacent peaks differ by only one charge unit, each peak becomes an independent measure of the parent mass. This redundancy makes signal-averaging possible over the peaks of a single spectrum with a consequent reduction in noise and an increase in the confidence level of mass assignment. We have considered in some detail the significance and utility of the peak multiplicity resulting from ES ionization and will report on its implications elsewhere. Here we will simply assert that we have developed a computer algorithm that carries out this signal averaging and "deconvolutes" the spectrum so as to present its message in terms of a singly charged peak whose m/z value equals the ion mass. Indeed, because the series of peaks produced by each species is coherent, the algorithm has been able to extract the data for each species when the injected solution comprised a mixture of two solutes. The limit on the number of solute species that can be accommodated by the algorithm will depend primarily on the resolution of the analyzer. It is to be noted that the values in the table for measured molecular weights were obtained by using this signal-averaging algorithm. The actual values were calculated from the known amino acid sequences [12]. Inspection of the table reveals that the agreement between measured and actual values of the molecular weight is remarkable. The error is generally within 0.2 percent and apparently decreases with increasing ion mass!

Our evident enthusiasm for ES ionization should not obscure the fact that other techniques have been developed which are also capable of producing ions from species of high molecular weight. Most of them constitute what might be termed the "energy-sudden" approach that has its roots in the original demonstration by Beuhler et al. that increasing the rate of heat

addition in the vaporization of non-volatile species can decrease the extent of fragmentation [13]. These methods are much less reversible than ES, producing much more entropy because they depend upon depositing a lot of energy in a short time on a small area of the surface on which the sample species is adsorbed, or in which it is dissolved when the surface is of a liquid matrix. The vehicles used for rapid delivery of energy include laser photons, fast atoms or ions, and radioactive elements, usually Californium-252. These techniques have succeeded in producing ions from peptides and proteins with molecular weights as high as 35000, and in one case have produced ions of Lysozyme clusters with masses of 71 kilodaltons [14, 15]. Unfortunately, the ionization efficiency goes down rapidly with increasing molecular weight and the ion currents for largest species are much smaller than those we have already obtained with ES. The energy-sudden methods do not provide the extensive multiple charging that we find with ES so they require analyzers with much higher mass capability. Sample preparation is generally more tedious and the ions formed usually have high degrees of internal excitation so that peaks are often substantially broadened by predissociation. More often than not there is a lot of chemical background noise due to matrix peaks and fragments of analyte. Many leads to the very broad literature on this and other facets of mass spectrometry will be found in the recent and remarkably comprehensive review by Burlingame et al. [16].

This report has been very much preoccupied with the advantages of ES ionization for mass spectrometric analysis of large molecules. We would be remiss if we did not remind the reader that its ability to produce multiply charged ions from large molecules in solution has many other implications for research. In the first place it is clear that the identity and distribution of the observed ions, and to some extent their state, must reflect conditions in the droplet at the time of desorption. Therefore, there is much to be learned about solution chemistry as well as the desorption process from insightful interrogation of the ions produced. In addition, these large, multiply charged ions, cooled internally by the free jet expansion that transports them into vacuum, would make fascinating subjects per se for optical spectroscopy studies. Although after mass slection the currents may be too small for probing ions "on the fly" they could be trapped in an ICR cell or an inert matrix and examined more leisurely. Photofragmentation studies of the kind already carried out in ICR traps with Fourier Transform mass analysis of the fragments would be particularly interesting as would fragmentation by scattering collisions at high energy. Surfaces would be most attractive as scattering targets because their high effective mass would mean that a large fraction of the incident kinetic energy would be available in the center of mass for exciting internal modes of the ion. In addition to being of scientific interest in themselves such fragmentation studies could make a most important contribution to the practice of tandem mass spectrometry of MS-MS in sequencing biopolymers. An exciting added attraction for such fragmentation of ions with multiple charges is the prospect of obtaining a multiplicity of daughter ions from each parent. In a non-beam context mobility studies of multiply charged ions in gases should prove interesting as would their nucleation properties in supersaturated vapors. The point is that large and complex ions carrying many charges are indeed something new under the scientific sun. To the extent we can believe that history will continue to repeat itself, such novelty will almost certainly provide many opportunities for stimulating and rewarding research.

IV. Prospectus

A common caricature holds that because of an obsession with ultimate simplicity physicists think even diatomic molecules are overpopulated with atoms. It was for experiments with beams of monatomic species that Otto Stern won his Nobel prize. Perhaps not surprisingly, therefore, that prize was officially in Physics, as were those awarded to his heirs apparent, Rabi, Kusch, Lamb and Townes. Thus, it seems highly probable that a canvas of those who know his name would identify Stern as a true physicist, to the manor born. Oh frailty, thy name is poll! His Chair at Hamburg was in Chemistry! His leading disciple, Isidor Rabi, was also fostered in that discipline. If once a chemist, always a chemist, then Stern would surely be delighted at what wonders his beams have wrought in the hands of fellow clansmen since he retired the scene. Perceptive kinfolk, applying his approach and perspectives to species ever more polyatomic, now stand atop the Nobel summit in his native science.

How now will the future reckon with these beams? Is biology the next arena in which they will exercise their powers? Wielded by sufficiently alert and able advocates will they coax the marvelously complex molecules of living systems into surrendering vital secrets? Answers to such questions, and to others not yet asked, await those patient pilgrims who persist along the wondrous path down which Stern has led us.

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