REVIEWS

Electrospray Ionization Mass Spectrometry: How It All Began*

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A brief history of the development of electrospray ionization mass spectrometry in the author's laboratory at Yale University with reference to its roots in the work of Rayleigh, Zeleny, and Malcolm Dole.

Key Words: Electrospray, ionization, mass spectrometry.

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Malcolm Dole's now famous paper that led to electrospray ionization was published in 1968.[1] That was the year after I had left the faculty of mechanical engineering at Princeton to join the faculty of chemical engineering at Yale where I continued my ten-year affair with big leaks in vacuum systems. Those leaks had turned out to be very powerful and versatile research tools because they could produce molecular beams with much higher energies and intensities than could the effusive sources with which Otto Stern and his disciples had earned half a dozen Nobel prizes in physics. Moreover, the supersonic free jets issuing from these leaks introduced their component gases to nonequilibrium steady states having combinations of temperature and density not previously dreamt of, let alone encountered. Those supersonic free jets subsequently ushered in the era of electrospray ionization (ESI) in mass spectrometry (MS) and are thus responsible for my being here today. (Jets also powered my transport on this trip to Austin but those jets were neither supersonic nor free!)

When I was told that I would have the honor of receiving the 2002 award from the ABRF, I realized that once again I was following in the footsteps of Csaba Horvath who received this award in 2001. Csaba had also preceded me by a year or two in becoming a chemical engineer at Yale. That sequence, together with subsequent events, lends credence to the notion that the loom of Fate might be weaving some preordained pattern! When I arrived at New Haven in 1967 Csaba was spending half his time in the medical school, working with Professor Seymour "Sandy" Lipsky on HPLC, then known as "high pressure liquid chromatography." The success of their efforts changed the meaning of "P" from *pressure* to *performance,* an achievement honored by Csaba's receiving this ABRF award last year. The subject of this year's award—electrospray ionization—has become a hyphen of choice in the practice of LC-MS. Because detection follows separation in that practice, it seems entirely appropriate, and consistent with classic Presbyterian Predestination, that today's award for an LC detection method should follow last year's award for LC separation itself! Moreover, the notion of an intervention by Fate is given still more credence by the fact that, unbeknownst to the Award Committee,

Csaba had played a key role in leading me into the paths of ESI-MS!

Never a very conscientious student of the literature, I didn't see Dole's paper when it appeared, but not long thereafter it was brought to Sandy Lipsky's attention. A mass spectrometrist as well as a chromatographer, Sandy was very much intrigued by Dole's ideas because they seemed to offer a way of using mass spectrometry to obtain precise values of molecular weight for large biomolecules like proteins. This cherished but elusive goal had long seemed a nearly impossible dream because all the then-known methods for ionizing any molecule depended on an appropriately energetic gas phase encounter between a neutral molecule and an electron, photon, or ion of some other atom or molecule. But large polyatomic molecules, including the complex and fragile species of biological interest, could not be vaporized without extensive fragmentation and decomposition. In 1966, when Dole first had the idea that led to his 1968 paper, there was no evidence of any possible solution to the vaporization problem. But in 1951 the first step had been taken along one of two paths that ultimately led to successful "soft" ionization methods, though it was not appreciated at the time. In that year Mueller discovered that when a sharp metal point was at a sufficiently high voltage in vacuo, the electric field at the tip of that point could be intense enough to extract an electron from a nearby gaseous atom or molecule to form a positive ion,[2] a discovery that led to his development of field ion microscopy.[3] The first use of such points as sources of ions for mass analysis was reported three years later by Inghram and Gomer,[4] but only with gaseous molecules. The ion currents were too small to stir up much interest, but they were larger than could be accounted for by the flux of molecules from the ambient gas into the region occupied by the point. Beckey then suggested that this apparent excess in ion formation rate could be accounted for by molecules that struck the surface of the wire at some distance from its tip and then arrived at the tip region by surface diffusion. In 1963 he showed that arrays of "whiskers" on thin wires could produce much higher currents than single needles and that nonvolatile species present on the surfaces of such whiskers could migrate to the tip by surface diffusion to be desorbed as intact ions. Beckey went on to develop this discovery into "field desorption ionization mass spectrometry" (FDI-MS) and in 1977 published a book on the subject.[5] Emitter electrodes comprising wires with dense arrays of whiskers are now available off the shelf at relatively low cost, but dosing them with sample and positioning them in the vacuum system are time-consuming and tedious tasks.

Moreover, ion currents depend so strongly upon both the temperature of the emitter and the applied voltage that substantial manual skill is required to find and maintain the right combination for a particular sample. That control function might now be made much less tedious by computers, but solving the control problem does not deal with an even more important difficulty. The ions from field desorption are released from high voltage into high vacuum. Consequently, their kinetic energies are so high that analysis of their masses required relatively large and expensive magnetic sector instruments. For these and other reasons, FDI-MS a la Beckey has never been widely practiced. However, from the perspective of history's hindsight one can argue that the ability of intense fields at the surface of a tiny charged droplet, which play a vital role in Dole's ESI, is simply an obvious extension of the discoveries by Mueller and Beckey on the nature of field ionization at a sharp tip, i.e., a surface with a very small radius of curvature. Maybe so, but it is also quite clear that nobody recognized these possibilities at the time, and that Dole arrived at the ESI approach by a logic that depended in no way on the field ionization ideas of Mueller and Beckey. In other words Dole was a true pioneer in that his ESI ideas were breaking brand new ground.

Fields intense enough to desorb solute ions at useful rates from a condensed phase can also be achieved without the array of very sharp needles or "whiskers" required by Beckey's approach. In 1972 Evans and Hendricks found that a high voltage applied to the surface of a nonvolatile conducting liquid in vacuo would produce a sharply pointed cone.[6] The field at the tip of such a cone was intense enough to desorb charge-bearing molecules and clusters of the liquid. If the liquid was a solution, the desorbed ions included solute species. These observations led to so-called "electrohydrodynamic ionization" (EHI) which was introduced to the mass spectrometry community in 1974 by Simons et al.[7] In EHI a sample solution is electrosprayed from a small bore tube maintained at high potential relative to the surroundings, just as had been taught by Zeleny and adopted by Dole. The difference is that in Dole's ESI, the droplets are dispersed into gas at near atmospheric pressure. In EHI, the sample solution is dispersed into vacuum. In that case the solvent must have a very low vapor pressure in order to avoid "freeze drying." But as Dole had recognized, evaporation of solvent is the sine qua non for the formation of solute ions from charged droplets and such evaporation requires a source of enthalpy. This dilemma has never been completely resolved in EHI because no satisfactory method has yet been found by which the enthalpy needed to vaporize sol-

vent from a droplet can be supplied to such a droplet in vacuo. Moreover, as in the case of FDI, any ions produced by EHI in vacuo have such high kinetic energies that magnetic sector instruments are generally required for their mass analysis. For these reasons EHI-MS has never become widely used despite the fairly extensive research that has been reviewed in detail by Cook.[8]

In sum, Malcolm's Dole's paper in 1968 was the seed which, after an extended period of germination, ultimately blossomed into electrospray ionization—ESI—one of the two "soft" ionization methods that have made the precision, sensitivity, and elegance of mass spectrometry readily available for the study of biomolecules and their reactions. The second of those two "soft" ionization methods is so-called matrix-assisted laser desorption ionization (MALDI), which was introduced by Karas and Hillenkamp in 1988 (see ref. 15) at almost the same time that ESI had begun to show its muscle. MALDI had also had a long germination period but was born of very different ancestry. A brief history of each of these techniques is summarized in the next two sections of this report. MALDI's story is told first even though ESI's was the first to begin.

THE ENERGY-SUDDEN METHODS

A completely different approach to the problem of producing intact ions from complex and nonvolatile molecules was embodied in what might be called the "energy-sudden" approach, based on an idea originally proposed in 1974 by Beuhler et al.[9] On the grounds of rate theory for unimolecular decomposition they argued that sufficiently rapid heating could vaporize complex molecules before they had time to decompose. In some proof-of-principle experiments on the vaporization of small peptides they showed that decomposition indeed decreased with increasing heating rates, but they never were able to heat rapidly enough to eliminate substantial fragmentation. The several techniques based on this idea include so-called pyrolysis mass spectrometry (PMS),[10] pre-MALDI versions of laser desorption (LD),[11] fast atom bombardment (FAB),[12] fast ion bombardment (usually called SIMS for secondary ionization mass spectrometry),[13] and plasma desorption (PD),[14] in which the sudden energy comes from the decay of a radioactive isotope, usually californium (^{252}Cf). PD was the first of these techniques to gain appreciable use. Its name stems from the idea that the energy released by the nuclear disintegration formed a small blob of plasma on the sample-bearing surface. As many as 30 or more analyte ions are ejected from that plasma when the analyte is dispersed in an appropriate matrix, typically nitrocellulose. 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 marker is provided by a back-scattered decay product of the Cf disintegration.

All these "energy-sudden" methods depend on the nearly instantaneous achievement of high-energy density in a sample dispersed on or in a solid or liquid surface. Thus they can be regarded as extensions of the rapid heating idea of Beuhler et al. They all could produce intact ions from many complex molecules including peptides and small proteins. However, their procedures were complex and awkward, and because their processes were highly irreversible, the yields of intact ions were very low. Even so, during the 70s and 80s they provided substantial accumulations of mass spectral data on a variety of complex, nonvolatile molecules, giving rise to a literature much too large for review or comment here.

By far the most important legacy of these energy-sudden methods is so-called matrix-assisted laser desorption ionization, or MALDI, introduced in 1988 by Karas and Hillenkamp.[15] Oddly enough, that was the same year that ESI-MS was first shown to be capable of producing intact ions from relatively large peptides and proteins. In MALDI, the analyte molecules are dispersed on a surface in a thin layer of matrix, usually an organic acid. The energy of an incident pulse of laser photons is absorbed mostly by the matrix to form a jet of matrix vapor that lifts analyte molecules from the surface and, by mechanisms still not well understood, transforms some of them into ions that are mostly singly charged. Because those ions are all produced at a well-defined location in an exceedingly short time, their mass analysis is most effectively achieved by TOF methods, as in the case of PD. MALDI is one of the two ionization methods for biomolecules that promise to dominate the MS scene for the foreseeable future.

ELECTROSPRAY IONIZATION

The other of those two dominant methods is electrospray ionization, the subject of the 1968 paper by Malcolm Dole and his colleagues[1] that Sandy Lipsky showed me in 1969. In fact, Dole had first proposed his electrospray idea two years earlier (1966) at the International Symposium on Macromolecular Chemistry in Tokyo. Those two communications comprise a testament to Dole's remarkable acumen in realizing that the production and behavior of highly charged droplets from solutions of nonvolatile solute species

in volatile solvents might produce intact gaseous ions of those species. Perhaps even more remarkable was the extent of his insight on the roles of ESI's several component processes. For example, he clearly recognized the need to disperse the charged droplets in a gas at relatively high (atmospheric) pressure. Such gas would provide the enthalpy required to vaporize solvent from those highly charged droplets. Moreover, collisions of the ions with molecules of that gas would reduce their initially high kinetic energies to those of the ambient neutral gas molecules, thereby avoiding the need for mass analyzers capable of accommodating high energy ions, e.g., magnetic sector instruments.

As most mass spectrometrists now know, the essence of Dole's idea was to take advantage of what happens during the evaporation of solvent from a droplet that has a net electric charge. In 1882 Rayleigh had analyzed the behavior to be expected of such a droplet and found that as the solvent evaporated the density of charges on the droplet surface would increase to a critical value, now known as the Rayleigh limit, at which Coulomb repulsion would overcome surface tension. The resulting instability would cause the droplet to break up into a plurality of offspring droplets.[16] Thirty years later John Zeleny made the first reported observation of this "Rayleigh instability" in pioneering experimental studies on the production and behavior of charged droplets carried out first at the University of Minnesota and then at Yale.[17] He passed a stream of conducting volatile liquid through a small-bore, thin-walled tube or "needle" (because it now often comprises a short length of hypodermic needle tubing) maintained at a high potential relative to an opposing counter electrode. The resulting intense electric field at the tube tip dispersed the emerging liquid into a fine spray of charged droplets. Zeleny was able to see that charged droplets did indeed break up as the solvent evaporated, just as Rayleigh had predicted. However, not until 1994, over a century after that prediction, were the fascinating details of such breakups revealed in elegant photographs by Gomez and Tang.[18]

Those photographs also clearly reveal how the intense field at the tip of the needle transforms the emerging liquid into a fine spray of charged droplets. In brief, as Figure 1 attempts to show, interaction between the forces due to surface tension of the liquid and those due to the imposed field results in the formation of a conical meniscus at the tube tip. That meniscus is known as a Taylor cone because its formation was predicted theoretically in 1964 by G. I. Taylor.[19] In actuality, Taylor's treatment applied to a nonconducting liquid for which there is no electric current and no liquid flow, but the name is still applied even when the liquid is conductor and a thin jet emerges from the tip of the cone.

As the result of another kind of instability, also first characterized by Rayleigh for uncharged liquids,[20] the interaction between viscosity and surface tension produces so-called "varicose waves" on the surface of such a liquid jet. Those waves grow in amplitude until they truncate the jet into a series of uniform droplets, as Figure 1 depicts. (That same kind of instability causes the breakup into droplets of a solid jet of water issuing from a garden hose or, on a smaller scale, the tiny stream of water from a tap that is incompletely turned off.) In the electrospray case, the droplets all have charges of the same sign (excess anions or cations, depending on the polarity of the applied field). Coulomb repulsion thus results in a divergence of their trajectories to form a conical "electrospray" of charged droplets.

Dole's scenario further assumed that the "offspring" droplets resulting from a Rayleigh instability would continue to evaporate until they too would reach the Rayleigh limit and break up into still smaller droplets. If the original solution were sufficiently dilute, a succession of such Rayleigh instabilities would ultimately produce droplets so small that each one would contain only one solute molecule. That lone molecule would retain some of its droplet's charge to become a free gas phase ion as the last of the solvent evaporated.

In the 1968 paper that described this scenario Dole also presented preliminary experimental results obtained in an attempt to translate his idea into practice. As taught by Zeleny, a solution of polystyrene molecules was infused through a small bore tube maintained at high potential relative to a counter electrode. Dole was always generous and conscientious in acknowledging the work of others, so it is curious that though he refers to Rayleigh's analysis of droplet instability he never mentioned Zeleny in any of his papers, even though his method of producing charged droplets seems to derive directly from Zeleny's papers. His account of the electrospray experiments in his autobiography simply says: "I got this idea from learning about the electrospraying of paint on to automobile bodies while working as a consultant for a paint company in Chicago."[21] Such "electrospraying" of paint consisted of maintaining a potential difference between the sprayer and the object being painted. The result was enough charge on the paint droplets to attract them toward the object being painted, thereby decreasing substantially the loss of paint to the surroundings by convection currents. Similar charging of droplets was practiced by crop dusting airplanes to decrease aggregation and increase adhesion of insecticides to the plants.
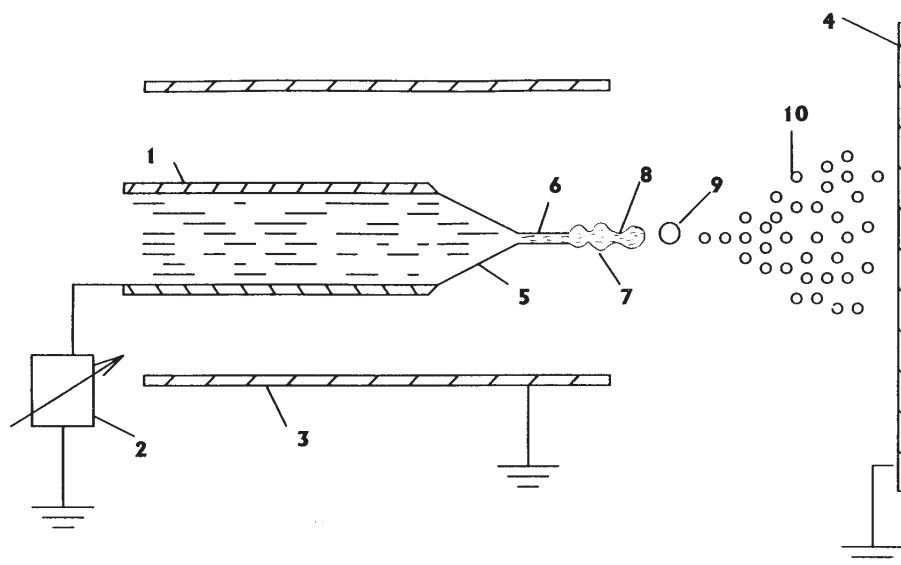
FIGURE 1

The mechanics of producing an electrospray. The injection needle (1) is maintained by a power supply (2) at a desired potential relative to the spray chamber wall (3) and counter electrode (4). The resulting field at the needle tip forms the emerging liquid into a conical meniscus (5) from whose tip flows a liquid jet with a diameter much smaller than that of the needle. Interaction between surface tension and viscosity produces varicose waves on the jet surface which grow in amplitude until they truncate the jet into a sequence of charged droplets with diameters slightly larger than that of the jet. Consequent to Coulomb repulsion, the droplet trajectories diverge to form a conical "electrospray."

But Dole had another problem to solve. The mass spectrometers then available, even the most expensive magnetic sector instruments, were unable to "weigh" singly charged ions with masses larger than about ten thousand Daltons. The oligomer ions of interest to Dole could have molecular weights up to a million or more and he had apparently not taken into account the possibility of extensive multiple charging, which had not yet been discovered. The problem he thus faced was how to "weigh" such large ions after they had been produced. Moreover, he found that the magnetic electron multiplier (MEM) on his Bendix mass spectrometer did not respond to his ES ions, which he explained as follows in his autobiography: "Although these ions had the same kinetic energy as light ions their velocity decreased in the ratio of the square root of their mass. We proved the mass limitation of the MEM in a separate experiment."[21] That comment strongly suggests that the ions Dole had produced from polystyrene oligomers did not have extensive multiple charging. Therefore, their mass/charge ratios were too high for available analyzers so he had to find another method for "weighing" them. That is how our free jet studies entered the picture.

We had shown that during such free jet expansions of mixtures comprising a very dilute solution of heavy "seed" molecules in a light carrier gas (e.g., argon in helium), the heavy seed species were accelerated like dust particles in a windstorm to almost the velocity that would be reached by the light carrier gas alone. Consequently, the translational kinetic energy of the argon molecules after expansion was higher than would be achieved in a jet of pure argon by a factor roughly equal to the ratio of the molecular weights of the two species (i.e., 10 in the case of argon accelerated by helium).[22] This acceleration effect was important to Dole because it seemed to provide a way to weigh the large polymer ions he hoped to produce. As shown in Figure 2 (from his 1968 paper) he placed a variable potential grid between the skimmer (that passed a collimated beam of ions from the free jet source into a vacuum chamber) and a sensitive galvanometer in a Faraday cage contained in that chamber. The only ions that could pass through the grid and reach the galvanometer
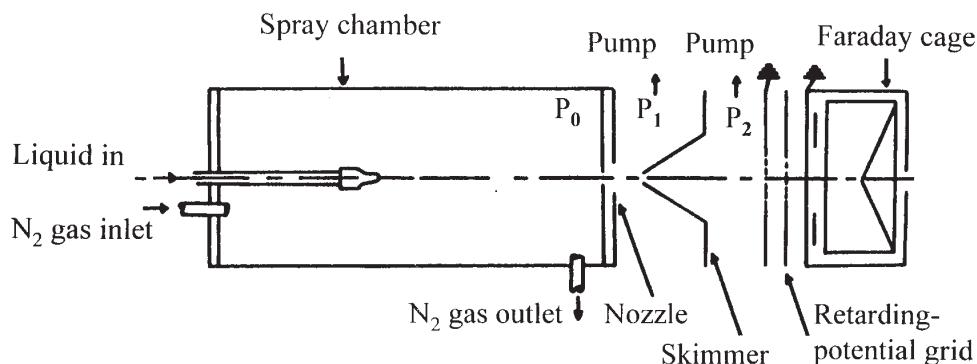
FIGURE 2

Schematic representation of Dole's original apparatus. The electrospray forms at the tip of the tube through which liquid enters the spray chamber concurrent with the nitrogen bath gas. Most of that leaves the chamber near the end plate containing the nozzle through which a small portion of the mixture of bath gas and ions enters the vacuum system in the form of a supersonic free jet. A small fraction of the ion-bearing jet gas passes though a skimmer into a second vacuum stage, thence through a retarding-potential grid into a Faraday cage connected to a galvanometer.

were those with a translational kinetic energy ($mv^2/2$) exceeding the product of the charge on those ions and the potential on the grid. The translational velocity v of the stopped ions was equal to the readily calculated velocity of the carrier gas. Thus, scanning the voltage applied to the grid should produce a current–voltage curve with a decrease in current to the galvanometer at every value of the voltage corresponding to the energy of an ion species in the beam. Figure 3 shows the curve Dole obtained with a fractionated sample of polystyrene having a nominal Mr of 51,000 at a concentration of 0.01 % by weight in 60:40 (v:v) benzene:acetone. Also reported were results with polystyrene having a nominal Mr of 411,000 and with a mixture of the two. The results showed a certain consistency with expectations but, possibly for reasons to be described, were not convincing enough to persuade other investigators to confirm or extend them.

With characteristic enthusiasm Sandy showed Dole's paper to Csaba. Noting the references to our Princeton papers Csaba told him that the John Fenn of those references had just moved to Yale so Sandy showed the paper to me. Always looking for new ways to apply our free jet technology, I was readily seduced by Dole's results and Sandy's enthusiasm. Thus began a friendship with Sandy that lasted until his death and a romance with mass spectrometry that will doubtless last until mine.

ELECTROSPRAY IONIZATION AT YALE

Early Efforts

A year or so after Sandy showed me Dole's paper I persuaded a new graduate student, Mike Labowsky, to repeat Dole's experiments in our much bigger vacuum system with much faster pumps than Dole had. We also had a better understanding of free jet expansions and realized that Dole had overlooked two of their features which were crucial in his experiments: (1) For the very large differences in molecular weight between the carrier gas and the seed species that he was using, there would have been a substantial amount of "slip" so that the actual velocities of his heaviest ions could have been as much as 50% lower than he had reckoned. (2) During the adiabatic expansion that occurs in a free jet the temperature of the gas nose dives. In the case of nitrogen, for example, at an axial distance downstream from the orifice of only ten nozzle diameters (ca. 1 mm in Dole's experiments), the absolute temperature of the gas drops to about 5% or less of its source value! Thus, the expanding gas rapidly becomes supersaturated with solvent vapor resulting from the evaporation of the ES droplets before the expansion. Ions are famously effective as condensation nuclei so that any ES ions in Dole's experiments must have been substantially resolvated to an
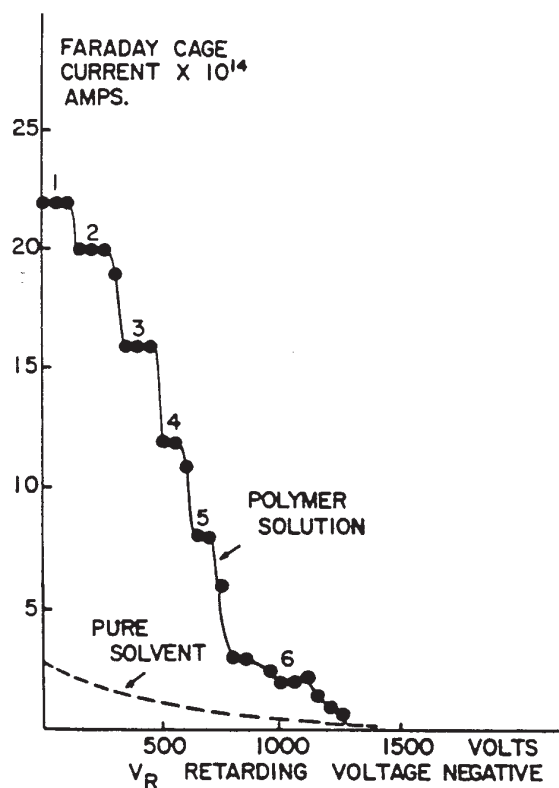
FIGURE 3

A current–voltage curve obtained by Dole when the electrosprayed solution comprised 0.005 wt percent each of polystyrene samples having molecular weights of 411,000 and 51,000, respectively. The solvent comprised 3 parts of benzene to 2 parts of acetone by volume. The distances from the needle to the first aperture, the first aperture to the second aperture, and the second aperture to the nozzle were, respectively 2, 3, and 3 inches.

unknown extent so their actual masses must have been much lower than Dole's measurements had indicated.

In order to avoid such resolution problems, Mike modified the Dole arrangement so that (with reference to Figure 2) the bath gas of dry nitrogen was introduced at the exit-nozzle end of the spray chamber. Consequently, drying gas flowed "upstream," counter-current to the flux of charged droplets and ions from the spray needle, leaving the spray chamber through an exit port at its "entrance end," i.e., opposite to the end containing the nozzle leading into the vacuum system. Thus, the solvent vapor from the evaporating droplets was carried out of the system so that the gas expanding into vacuum through the nozzle was free of solvent and any other "junk" that was not ionized. This counter-current flow of bath gas solved the problem of ion resolvation during the free jet expansion, greatly reduced system fouling, and has become a feature of most successful ESI-MS systems. It will be described later in more detail.

One can also avoid resolvation of ions during expansion by raising the temperature of the ion gas mixture to a value high enough so that after free jet expansion the gas temperature remains above "the dew point" for the ions. This "fix" was demonstrated by Chowdhury et al.[23] and has been incorporated in some commercial instruments. It has had limited use because unlike a counter-current gas flow, it does not eliminate uncharged "junk" from the gas entering the vacuum system. Consequently, more frequent system cleaning is required.

As already mentioned, Dole's paper apparently failed to persuade other investigators to confirm his experiments which, though simple in principle, were really very difficult and demanding in practice. For example, large macroions incident on the first dynode of a multiplier do not produce secondary electrons unless their incident velocities are very high, either because of multiple charging or high accelerating voltages. As implied by his statements in his autogiography[21] it seems highly likely that Dole's ions did not have many charges. Consequently, in his later experiments he resorted to ion mobility measurements to characterize his macroions.

In 1969 at the age of 66, just two years before mandatory retirement at Northwestern, Dole accepted an offer to become Welch Professor of Chemistry at

Baylor University in Waco, Texas where he was assured there would be no mandatory retirement as there was at Northwestern. At Baylor he continued his electrospray experiments using mobility measurements rather than mass analysis to characterize the ions. He presented a short paper on that work at the 33rd Annual Meeting of American Society of Mass Spectrometery (ASMS) at San Diego in 1985, where I also gave a paper that included a discussion of the cooling and resultant resolution that occurred in free jets. Dole came up to me afterward to introduce himself and to express his gratitude for finally understanding why there didn't seem to be any reasonable relation in his experiments between the size of the molecules and the mobilities of their ES ions. He said "Now I realize that we have been measuring the mobilities of highly solvated ions!" That was our only face-to-face encounter but we subsequently exchanged several letters and had a cordial relationship. I was delighted when he was later able to attend the first ASMS Workshop on ESI-MS in Chicago. I didn't see him there because I had not been invited but by that time the "electrospray revolution" was well on its way, so he had the satisfaction of realizing how fruitful his ideas had become before he passed on to his ultimate reward some months later. He would have been amazed if he had known that by 2001 the number of papers *per year* on ESI in the archival journals would reach 1500 and still be growing![1]

There was a hiatus in ESI studies at Yale after 1975 when, for understandable reasons, Mike Labowsky got discouraged. The retarding-potential method of determining ion mass was most exasperating. The only way we could measure the very small currents was with an old vibrating-reed electrometer that on its best days was crankier than a model T

[1]In retrospect I would have been wise to follow Dole's example and leave Yale before I became subject to mandatory retirement in 1987, the last year that it was allowed and just as the electrospray research was becoming most interesting. For the next six years Yale bureaucrats did everything they could to prevent that research from continuing, though they happily allowed the grant money to be spent and eagerly pocketed all the funds allowed in the grants for indirect expense. The most unfortunate consequence was that for the last five of those years Yale refused to provide the necessary space to install a new triple quadrupole mass spectrometer, which had not only been funded by NIH but which Yale had agreed to provide in *several* research proposals for which funds had been granted! That instrument didn't get used until I brought it to VCU in 1994 when I left Yale. I often wonder how much more we might have accomplished had we been able to use it during those five lost years.

Ford! Mike had become interested in the evaporation behavior of the cloud of ES droplets and thus in the evaporation and combustion of a cloud of interacting fuel droplets—an important and challenging practical problem of interest to several of my colleagues in the department. He developed some unique computational techniques and published several important papers on this classic problem that became the main basis for his thesis.

Then, in 1982, Masamichi Yamashita, a young scientist I had met in Japan, came to my lab as a postdoc. When he arrived, "Gado" as he was known, asked for some suggestions about what he might work on. I mentioned that it might be interesting to take another look at ESI, but instead of trying to make ions out of big molecules, to explore whether the process would work with molecules small enough for their ions to be analyzed with a small quadrupole mass spectrometer that was a detector in some of our molecular beam experiments and which had an upper limit for mass/charge ratio of only 450. Gado liked the idea and off he went. Not only was he an extraordinary experimentalist, with sound instincts and magic hands, but he knew everything from the biology of sea urchins to the practice of digital electronics (already in 1982!). In a relatively short time he assembled what was really the world's first ESI mass spectrometer from the bits and pieces of molecular beam apparatus in our precious "junk pile." (My Scottish heritage of "waste not, want not" makes it very difficult for me to throw anything away that might have some conceivable use!)

That first apparatus is shown schematically in Figure 4 and illustrates the arrangement for providing a counter-current flow of drying gas. We first tried electrospraying methanol–water mixtures and obtained spectra showing peaks corresponding, for example, to protons solvated by various combinations and numbers of methanol and water molecules that could be varied widely simply by adjusting the flow rate and/or temperature of the counter-current drying gas. We then added some solutes to the solvent and were rewarded with the spectrum shown Figure 5 *(top panel)*. It was obtained with a solution of several tetraalkyl ammonium (and one phosphonium) halides at concentrations of a few parts per million. These results were especially gratifying because those solutes cannot be vaporized without extensive, even catastrophic, decomposition. Beginning to get excited, Gado dissolved a vitamin B tablet in methanol–water and obtained the spectrum in Figure 5 *(bottom panel),* which shows a clearly defined peak for intact ions of every species in that tablet (i.e., with an Mr value under 450.)

The spectra in Figure 5 were exciting to us because they clearly demonstrated that ESI could pro-
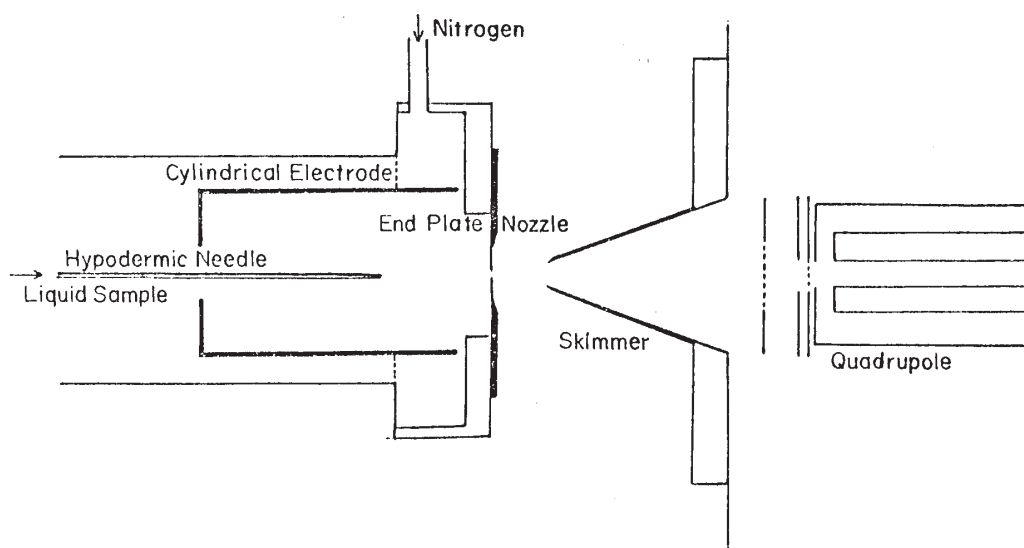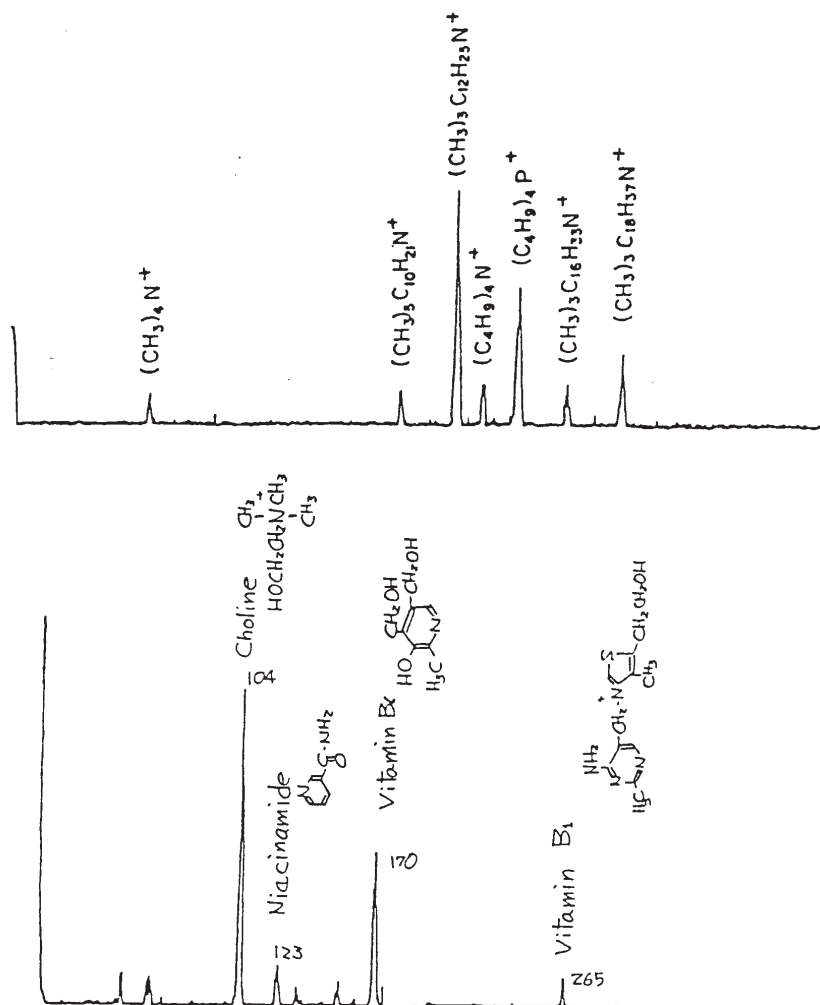
FIGURE 4

The first electrospray mass spectrometer built at Yale. Sample solution was sprayed from the hypodermic needle into a counter-current flow of dry nitrogen. The needle was at high potential relative to the cylindrical electrode and the end plate containing the orifice into the vacuum system. A center portion of the resulting free jet passed through the skimmer into a second vacuum chamber containing a quadrupole analyzer.

duce intact ions of each of several species in a plurality of fragile molecules in a dilute solution while avoiding both fragmentation and interference between them. The spectra also raised second thoughts about the mechanism of ionization. We had been tacitly assuming that Dole's charged residue model (CRM) accounted for the formation of the large ions he had found as well as the small ions that we had found in the results just described. In our early experiments, as well as in Dole's, there were always many more analyte (solute) molecules than charges in the ES flux. Consequently, as our results accumulated, we become increasingly uneasy about the adequacy of the CRM. The spectra in Figure 5 heightened that concern because we could not understand how the sequence of Rayleigh instabilities could produce, for each of several species in the same solution, singly charged ultimate droplets containing only one molecule of that species. Moreover, if the CRM model was to explain the results, the relative number of those droplets for each species would have to be in direct proportion to the relative concentration of that species! How could the droplet know how to program its sequence of Rayleigh instabilities so as to produce a distribution of ultimate droplets such that the number of those ultimate droplets containing one molecule of any species X is always directly proportional to the concentration of species X in the original

solution? Of course that would happen if the droplet subdivision continued until all the droplets contained only one solute molecule, but that scenario somehow seemed unlikely, especially when rapid evaporation from all droplets was occurring from the start. Moreover, if the subdivision process were to produce ultimate droplets for each ionizable species in a solution, one should then always find a spectral peak for every species in such a solution, and that just doesn't happen.

These conceptual problems seemed to be resolved when we learned about some papers by Iribarne and Thomson, meteorologists at the University of Toronto.[24] Interested in the possibility that charged droplets of sea water might be a source of some of the ions found in the atmosphere, they carried out experiments showing that evaporation of charged droplets could indeed produce gas phase ions of solute species in those droplets. The charged droplets in those experiments were produced by pneumatically nebulizing a conducting liquid. In such nebulization the droplets become charged as a result of statistical fluctuations in the distribution of anions and cations among those droplets, so that roughly equal numbers of positively and negatively charged droplets are formed. In their first experiments only aerodynamic forces were used to nebulize the liquid, as in a perfume atomizer. Then they found that 3500

FIGURE 5

Early mass spectra obtained with the apparatus shown in Figure 4. The spectrum in the *upper panel* resulted when the sample solution contained one quarternary phosphonium halide and six quarternary ammonium halides at concentrations from 2 to 10 ppm in 50:50 methanol:water. The spectrum in the *lower panel* was obtained with an extract of a vitamin B tablet, also in 50:50 methanol:water.

volts applied to an "induction electrode" (a centimeter away from the nebulizing zone) would result in all the droplets—and the ions from their evaporation—having the same sign, positive or negative, depending on polarity of that electrode. Iribarne and Thomson called their technique atmospheric pressure ion evaporation (APIE), a name which literally refers more to the mechanism of ion formation from charged droplets than to the method of producing the droplets. Moreover, both the earlier electrospray ionization (ESI) of Dole and the later thermospray ionization (TSI) of Marvin Vestal[25] also depend upon vaporization of charged droplets, but their names do not identify any ion-forming mechanism. The essential differences between these three "spray" techniques consist mostly in the way the charged droplets are produced. Therefore, it seems more appropriate to give them names that relate to those characteristic differences, i.e., ESI for electrospray ionization, ASI for aerospray ionization, and TSI for thermospray ionization. These names relate specifically to the way droplets are produced in terms of the kind of energy used to produce them, (i.e., electrical, aerodynamic, and thermal) and will be used hereinafter.

The ASI technique of Iribarne and Thomson has never become has as widely used as ESI. However, their ion evaporation mechanism (IEM) for ion formation from charged droplets, set forth in their papers, was a milestone contribution and has become a formidable competitor for Dole's charged residue model (CRM) with each one having its champions. The feeling in this corner is that under most circumstances the IEM is more consistent with more experimental observations than is the CRM. However, sometimes the CRM seems more likely to apply, especially in the case of very large analyte molecules.

A bit more needs to be said about TSI, the thermospray ionization technique introduced by Marvin Vestal and his colleagues beginning in 1978.[25] The last of the charged droplet sources to appear, TSI became the first to be widely used, in part because of its effectiveness and in part because it was the first to become available commercially as an add-on to existing mass spectrometers. This sequence of events would seem to fulfill that passage in the scripture which says, "The last shall be first." (That passage continues by saying, "And the first shall be last," which apparently is being fulfilled by ESI because it was the first to be introduced and now would seem likely to be the last of the spray techniques to be abandoned, if it ever is.)

TSI operates by passing analyte 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, e.g., as in ASI or a perfume atomizer. The result is 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 as in ASI. Evaporation of the droplets in the superheated vapor brings about the sequence of Rayleigh instabilities common to all the techniques based on charged droplets. That sequence leads to the formation of gaseous ions by either the CRM of Dole or the IEM of Iribarne and Thomson. The mixture of ions in vapor flows past an aperture through which ions of the desired polarity are driven by an applied field into the vacuum chamber housing a mass analyzer. For several years in the 1980s, TSI sources became the preferred hyphen in LC-MS, but they have now been almost completely replaced by ESI sources.

Meanwhile, we were achieving ever better results with ESI as we climbed the learning curve, and Sandy Lipsky was keeping in close touch with our progress.

He was a good friend of Brian Green of VG Analytics, a British manufacturer of mass spectrometers that has since evolved into what is now Micromass, Ltd. Brian would stop by New Haven and visit Sandy every few months and on most of those visits Sandy brought him by our laboratory. As our results with small molecules began to get better and better, Brian became more and more intrigued and asked us if the technique would work with larger molecules. We said we thought it might but couldn't be sure because our little quadrupole analyzer had an upper limit of about 450 for mass/charge ratio. Brian then arranged for VG to lend us a used quadrupole analyzer that could weigh singly charged ions with mass/charge ratios up to 1500 or so. That instrument was incorporated in a new system for which much of the design and assembly was done by Craig Whitehouse. Craig had worked in Sandy's lab after finishing college and Sandy urged me to take him on for graduate study in chemical engineering. The design of this new system is shown schematically in Figure 6, and has features that deserve comment.

In light of the results obtained with Gado's machine, and with Csaba and Sandy hovering in the background, we increasingly viewed ESI as a possibly more potent hyphen in LC-MS than the already widely used TSI. An electric field sufficiently intense to disperse analyte solution emerging from the spray needle into a fine spray required a potential difference of up to several kilovolts between that tip and the orifice leading into the vacuum system. Thus, one was faced with a trilemma in LC-MS operation: Either (1) the LC system would have to be maintained at several kilovolts above ground, (2) the MS would have to be maintained at several kilovolts "below" ground, or (3) the power supply would require enough capacity to maintain the required potential difference in spite of the leak to ground of current through the conducting path comprising the flow of liquid from the LC. Options (1) and (2) were not feasible but option (3) was being used in some systems. We then had a wild idea which turned out to be a very effective solution to this problem. We would replace the simple orifice into the vacuum system by a glass capillary tube as shown in Figure 6. That glass tube was metallized at each end to provide an electrical contact. The inlet end of the capillary would be maintained at the required potential below ground so the ions entering the tube would be in a potential well. We guessed, correctly as it turned out, that the fairly high velocity flow of bath gas through the glass (dielectric) tube into the vacuum system could drag the ions out of that potential well up to whatever potential might be desired at the metallized exit end of that tube. It worked like a charm. Indeed, the gas flow could raise the ions to an exit potential of ten or more kilovolts, sufficient to inject
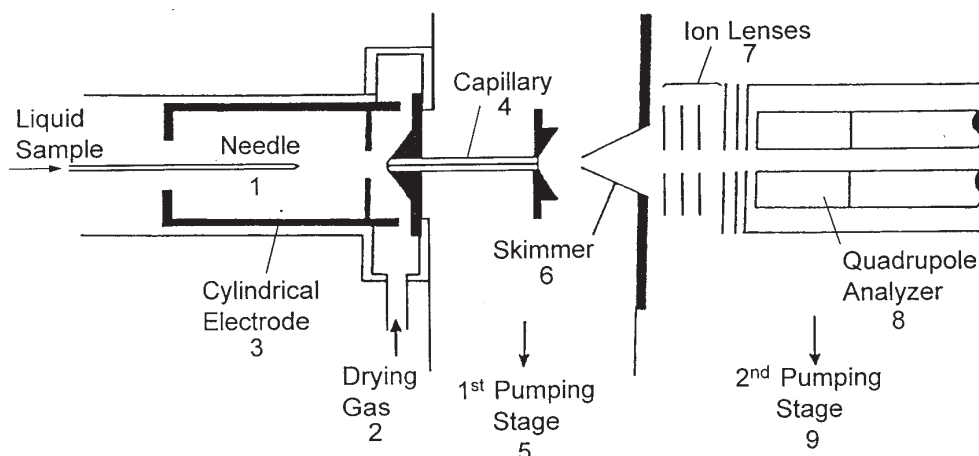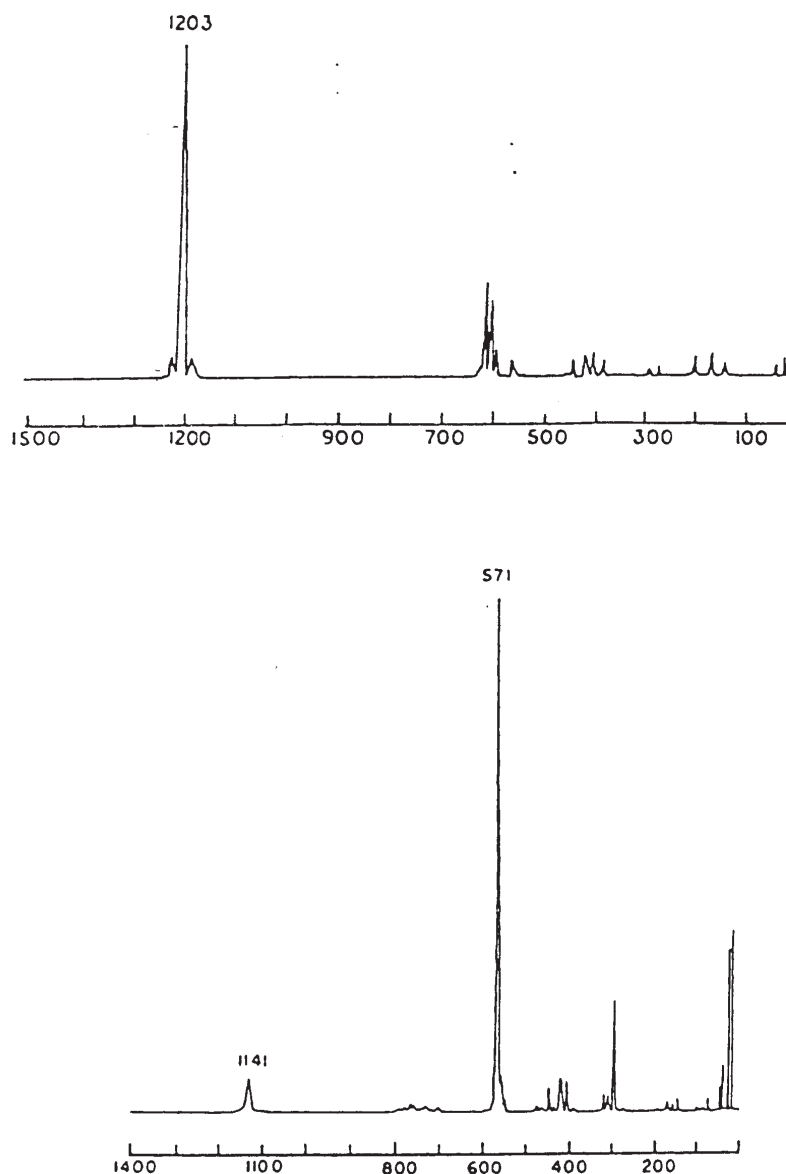
FIGURE 6

Schematic diagram of the second ESI-MS apparatus. It features the same counter-current flow of drying gas shown in Figure 4 which excludes solvent vapor from the ion–gas mixture that enters the vacuum system by way of a free jet expansion. A novel feature of this apparatus is that the ion-gas mixture enters the free jet expansion at the exit of glass capillary 4 rather than through the simple flat-plate orifice of Figure 4. Each end of that capillary is metallized so that it can be maintained at any desired potential. Thus, the field required to "electrospray" the sample solution can be achieved with the spray needle at ground, while the metallized entrance of the capillary is at the required potential "below" ground. The ions entering the capillary are then in a potential well. The rapid flow of gas then drags the ions out of that potential well to any desired potential at which the metallized exit end of the capillary is maintained. Thus, all external parts of the apparatus can be at ground, posing no hazard to an operator.

the ions into a magnetic sector analyzer. At the same time, all exposed external parts and surfaces of the instrument could be maintained at ground potential, thereby posing no hazard to an operator.

Eager to flex our muscles with the new apparatus we tried it out with a couple of cyclic peptides provided by some friends in the medical school, cyclosporin A and gramicidin S with molecular weights of 1184 and 1141, respectively. Figure 7 *(upper panel)* shows the spectrum obtained with a solution of 1.0 g/L of cyclosporin A in 85:25 (v:v) acetonitrile:water. The dominant peak at 1203 corresponds to singly protonated parent molecules with one water of hydration. The small satellite peaks on either side are due to ions with one more and one less water molecule. The small triplet peak near mass 600 is from doubly protonated ions of the same species. (The right to left increase in mass/charge ratio is a quirk of the available chart recorder.) Figure 7 *(lower panel)* shows the spectrum for 1.0 g/L of gramicidin S in 50:50 (v:v) methanol:water. Here the peak is much larger for ions with two charges rather than one. These differences can be understood in terms of the IEM mechanism and the much greater

hydrophobicity of cyclosporin. A molecule of the latter can be more easily removed from an aqueous environment than can one of hydrophilic gramicidin.

Peaks for doubly charged ions also dominated in similar spectra for the peptides bleomycin and substance P. In the spectra for renin substrate and insulin chain B we found evidence of triple protonation. This propensity for multiple charging was quite provocative because the effective mass range of any analyzer increases by a factor equal to the number of charges per ion. In order to explore the extent to which such multiple charging can occur, and to identify and evaluate the controlling factors, we carried out an extensive study of ES ion formation with poly (ethylene glycol) (PEG) oligomers. Samples with a range of nominal molecular weights (Mr values) were kindly provided by Union Carbide and Chemical Corp. Each one comprised a mixture of oligomers with a Gaussian-like distribution of Mr's having an FWHM roughly 15% of the nominal Mr of the most abundant oligomer. These species retain their chemical and structural similarity over a wide range of Mr values. It is known that Na^+ ions bind to the oxygen atoms in

FIGURE 7

Electrospray mass spectra obtained with the apparatus shown in Figure 6 for two small cyclic peptides. In the *upper panel,* for 0.1 g/L of cyclosporin A in 85:25 acetonitrile:water, the dominant peak at m/z =1203 corresponds to a singly protonated parent molecule with one water molecule of solvation. The tiny satellite peaks on either side represent ions with one more and one less water molecule. The much smaller triplet peak near mass 600 comprises doubly charged ions of the same species. The other small peaks are due to impurities. The *lower panel* is the spectrum for 0.01 g/L of gramicidin S in 50:50 methanol:water. Here, the dominant peak is for the doubly charged ion. The differences between these two species can be understood in terms of the ion evaporation model. Much more hydrophilic than cyclosporin, gramicidin needs two charges for the droplet surface field to overcome the ion's affinity for water.

the oligomer chain with an energy of 2.05 eV and there is always enough sodium around so that spectra of samples from the manufacturer (Union Carbide and Chemical) comprised oligomers with varying numbers of Na^+ adducts that increased with increasing molecular weight. (However, one can change these adducts by appropriate additions to the sample, e.g., to K^+ by adding KOH or KCl.) Figure 8 *(left panel)* shows mass spectra for PEG samples having nominal Mr values from 400 to 3350 for which the individual peaks are clearly resolvable in the original spectra and correspond to oligomers with varying numbers of Na^+ adducts. Figure 8 shows spectra for samples with nominal Mr values of 8000 and 17,500 wherein the peaks are much too close together to be resolved (with our analyzer) because of the superposition of a wide distribution of oligomer sizes on a wide distribution of charge states for each oligomer. The reviewer of our first paper on these results dismissed them outright, saying that those spectra were "not mass spectra but were due to dirt in the system!" However, we have found over the years that for polymers like these, one can correctly and usefully assume that the m/z value at the maximum or peak value of the envelope of the overlapping peaks is a reasonably reliable estimate of the most probable m/z value for the most abundant oligomer in the sample. We used this assumption to great advantage in a later study which showed that intact ions could be obtained from PEG oligomers with Mr values of at least 5,000,000![27] Others have since shown that ESI can produce intact ions of species with molecular weights of over 100 million!

The message of these PEG experiments was clearly that, to study the ESI-MS behavior of large molecules, we needed samples of pure compounds in which all the molecules had the same molecular weight. Obvious examples of such compounds are natural peptides and proteins which, for many other reasons, of course, are far more interesting and rewarding subjects of study than synthetic polymers. We began to get promising results with such compounds material in late 1987 and presented the results shown in Figure 9 in June 1988 at the 36th ASMS Conference on Mass Spectrometry and Allied Topics in San Francisco. Inspection of those spectra show that each peak in the spectrum for any one species differs from an adjacent peak only by one adduct charge, usually a proton in the case of these peptides and proteins. Therefore, each peak becomes an independent measure of the mass of the parent molecule. Armed with this realization, Matthias Mann, then a graduate student in our group, quickly worked out a couple of algorithms by which a computer could rapidly transform a very complex spectrum into the

spectrum that would be expected if each species had given rise to a single ion with one massless charge. The m/z value for that peak is thus the Mr value for that species. Figure 10 shows the result of that transform for the spectrum of cytochrome C in Figure 9.

There were only a handful of people at the session of the ASMS meeting at which we presented these results. Nor were there many questions or comments from the floor, but those results began what has become a flood of papers and have since been called by some, "the beginning of the electrospray revolution!" Some idea of the dimensions of that flood can be gained from Figure 11, which was prepared by my good friend and former colleague, Prof. Juan de la Mora at Yale, and shows how the publications on ESIMS have been increasing with time, reaching some 1500 per year in 2001! Moreover, those numbers reflect only a small fraction of the total ESI-MS activity, most of which is being carried out by the pharmaceutical companies and is not published.

However, not everyone was impressed. The reviewer of a reputable journal to which we submitted a manuscript on the results with poly(ethylene glycols) refused to approve publication on the grounds that the spectra clearly showed that the method was worthless! In the first place, the ion currents were distributed over so many peaks that signal/noise ratios would inevitably be too small. Moreover, the multiplicity of peaks for each species would make the spectra of any mixture hopelessly complex and uninterpretable! But as I myself have done all too often, that critic underestimated the power of the computer. (In fairness I should admit that that manuscript he saw didn't include any discussion of the algorithm that had not yet been worked out, but the fact that such spectrum could be obtained at all was surely worth publishing at that time.)

Funding agencies were generally somewhat less than enthusiastic in response to our proposals. For example, when we submitted one for research to extend the results shown in Figures 4 and 5, the reviewer simply said, "It is impossible for this man to obtain these results with his equipment!" I am reminded of the slogan once promoted by a company which said, "The difficult tasks we can finish quickly. The impossible ones take a little longer!"

In view of the flood of papers on ESI-MS revealed in Figure 11, the impossibility of summarizing what they have taught would clearly take much more than a little longer. Consequently, I will stop here with a final word of appreciation for the honor being chosen to receive the ABRF Award and the chance to reminisce about my adventures with Malcolm Dole's brain child. I would especially emphasize the extent of my
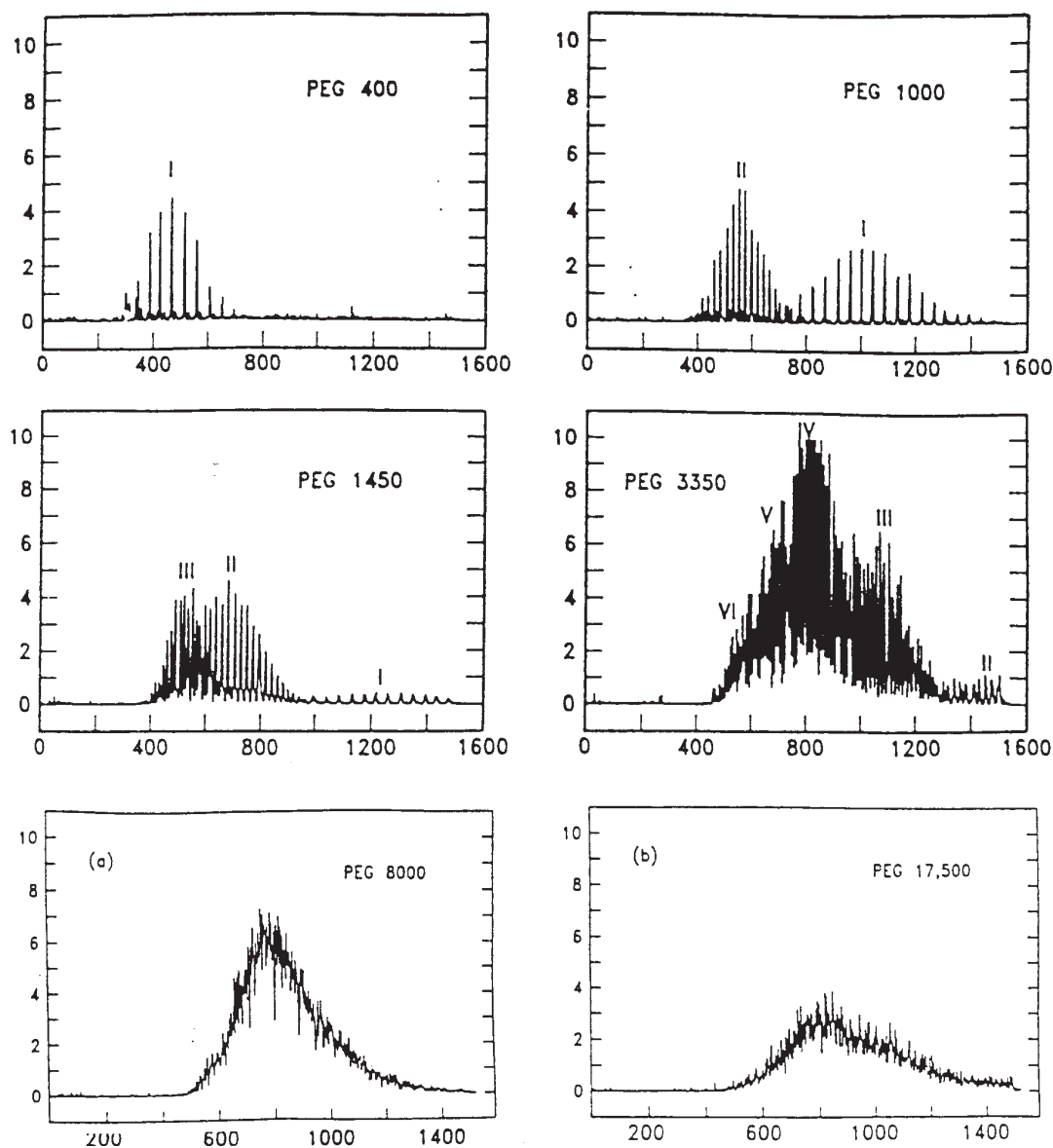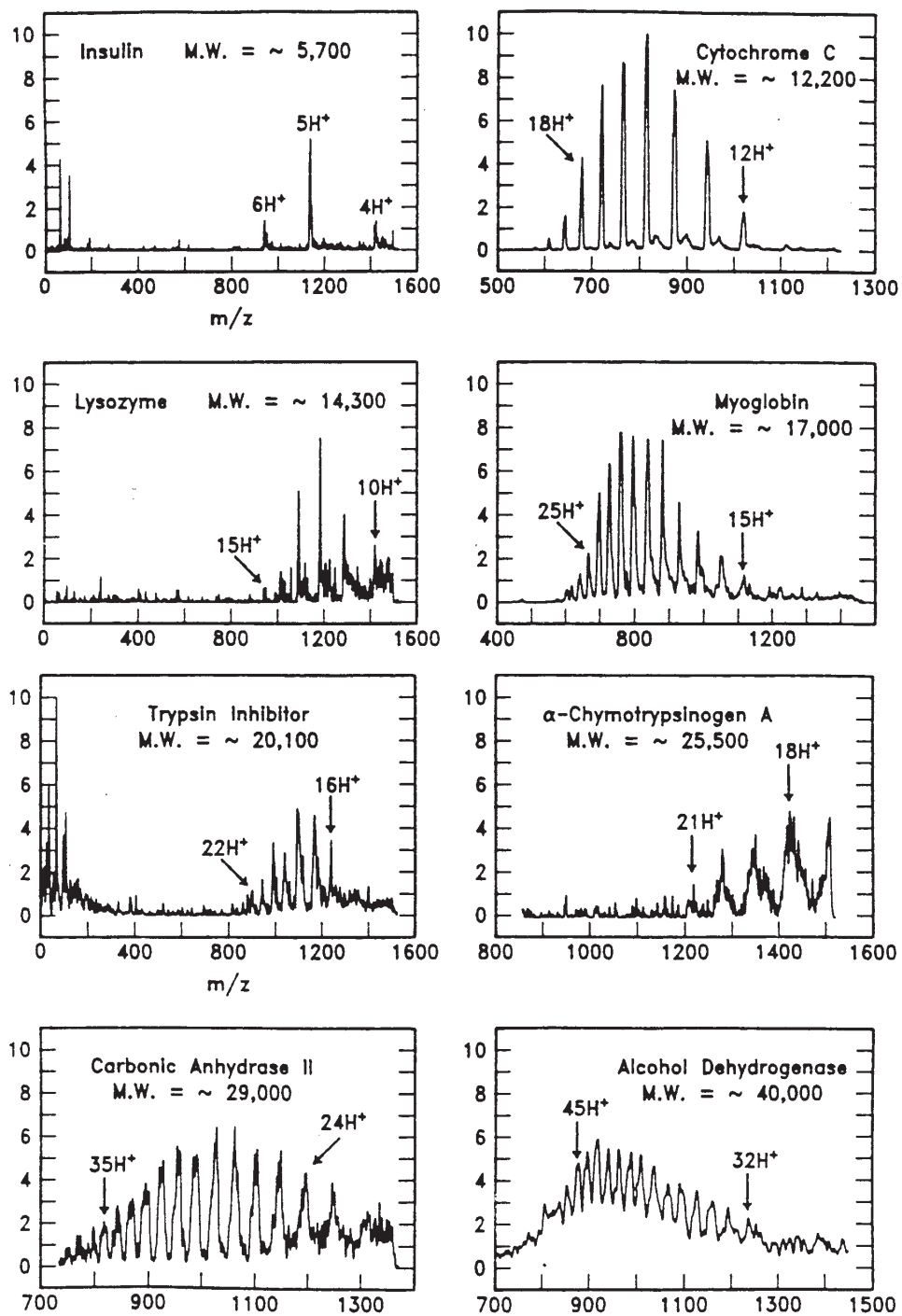
FIGURE 8

Electrospray mass specta obtained with the apparatus shown in Figure 6 for poly(ethylene glycol) samples of varying sizes. The nominal Mr is for the most abundant oligomer in a sample with a Gaussian distribution of Mr values. The increase in peak congestion with increasing Mr is due to the superposition of an increasingly broad distribution in charge states on an increasingly broad distribution of Mr values in the sample.
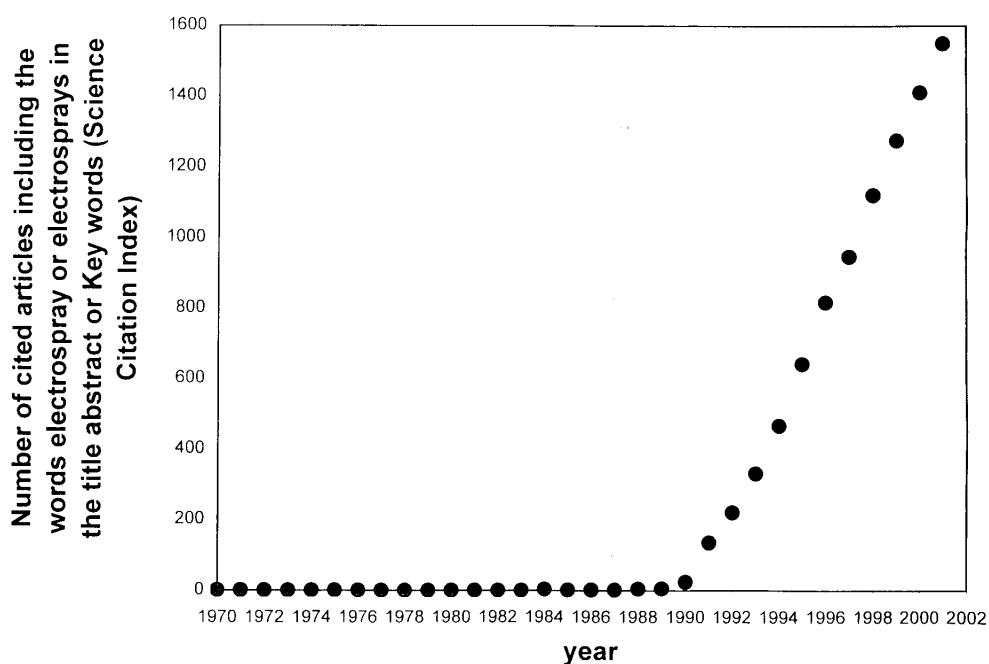
FIGURE 9

Electrospray mass spectra for various peptides and small proteins with Mr values up 40,000. These results precipitated the "electrospray revolution."
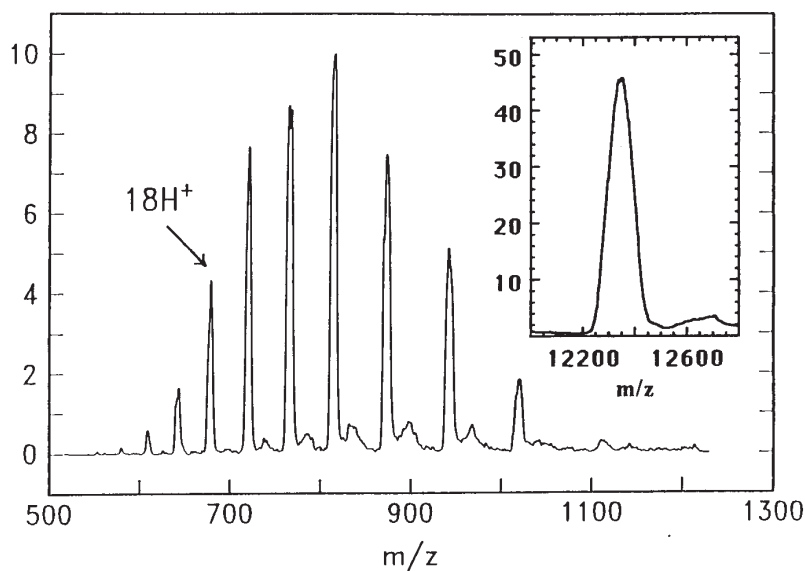
FIGURE 10

The electrospray mass spectrum is the one for cytochrome C shown in the uppermost right hand panel of Figure 9. The *inset* shows the transform of that spectrum by the Mann algorithm. It comprises the single peak that would have been obtained if all the ions comprised a parent molecule with a single, massless charge. Thus the *m/z* value for the peak is equal to the Mr value of the protein. Note that the transformed peak has almost five times the height of the highest peak in the spectrum (the ordinate scales are the same for both the spectrum and the transform). Moreover, the signal/noise ratio is much higher.

FIGURE 11

A graph showing the rapidly increasing activity in electrospray ionization mass spectrometry after the results shown in Figure 9 were published. The ordinate values (from the *Science Citation Index*) are for the number of papers published *each year* in which the word "electrospray(s)" appears in the title, abstract, or list of key words.

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