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Desorption of Ions from Rat Membranes: Selectivity of Different Ionization Techniques

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Complex lipid biomarkers, including phosphatidylcholines, cerebrosides and sulfatides, are shown to be desorbed intact from rat brain myelin and rat liver microsomes by liquid secondary ion mass spectrometry, by plasma desorption and by laser desorption. Different polar lipids are favored by the different desorption techniques and as negative or positive ions. These selectivities support current theories about ionization for the different techniques.

INTRODUCTION

In a previous communication[1] we noted that intact molecular ions of polar lipids could be desorbed directly from lyophilized cells and membranes and analyzed in a mass spectrometer. The desorption techniques which we used included fast atom bombardment (FAB) or liquid secondary ion mass spectrometry (SIMS), laser desorption (LD) and plasma desorption (PD). Such techniques yield interpretable and characteristic spectra of cells and membranes without involving the solvent extraction, separation and chemical derivatization procedures traditionally employed for analysis by gas chromatography/mass spectrometry (GC/MS).[2] At the same time, we suggested that desorption mass spectra could provide more structurally significant information on biomarkers than the direct analysis of cells and membranes by pyrolysis mass spectrometry.[3,4]

Subsequently, we focused our attention on the FAB desorption of polar lipids from whole, lysed bacterial cells, in order to determine whether the resulting mass spectra could be useful in distinguishing and/or identifying bacterial species. Initially we examined the negative ion FAB mass spectra of cells from three Gram-negative (*Escherichia coli*, *Pseudomonas fluorescens* and *Proteus vulgaris*) and three Gram-positive (*Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus*) bacteria.[5] Pseudomolecular $[M - H]^-$ ions were observed for phosphatidylethanolamine (PE), phosphatidylglycerol (PG), lysylphosphatidylglycerol (LPG), phosphatidylinositol (PI), diphosphatidylglycerol (DPG, cardiolipin) and diglycosyldiglyceride (DGDG) contained in the cell walls. Relative abundances of the molecular ions reflected the fatty acyl distributions of each bacterial sample as well as the predominance of particular classes of phospholipids. Thus, the mass spectra of Gram-negative bacteria were dominated by molecular ions of PE, the mass spectra of Gram-positive bacteria revealed primarily PG ions, and the distribution of these and other phospholipid ions yielded patterns distinctive for each bacterial species. We subsequently developed a small library of negative ion FAB mass spectra for a number of Gram-negative and Gram-positive species, and employed linear regression techniques for identifying 'unknowns' within that group (including different strains) and assessing the composition of mixtures of cells.[6]

We then noted[7] that the positive ion FAB mass spectra of whole, lysed cells provided an additional dimension for characterizing bacterial species. While protonated (MH^+) molecular ions are observed, fragment ions from the loss of the polar head group are also observed, are more abundant than for the negative ion case, and are characteristic for each phospholipid class. Using linked scanning of the magnetic field (B) and electrostatic analyzer voltage (E) on a double-focusing instrument, we have exploited constant neutral loss (CNL) mass spectra of lysed cells to enhance both selectivity and specificity for particular phospholipids and to characterize minor phospholipids (e.g. monomethylphosphatidylethanolamine, MMPE) peculiar to specific bacterial species. Thus we concluded that an effective protocol for computer-aided rapid identification of bacteria would consist of a combination of library matching of normally scanned negative ion FAB mass spectra and CNL positive ion searches to reduce the number of library candidates.

In this paper we have turned out attention to the desorption of ions from mammalian membranes. In

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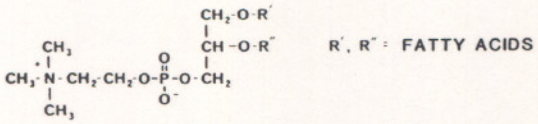
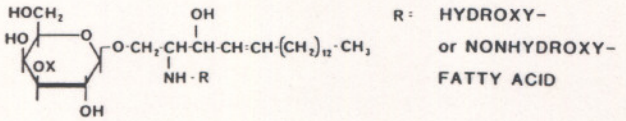
addition to the phospholipids examined in bacterial cells, membranes of nerve cells also contain cerebrosides and sulfatides (see Scheme 1). Thus, this broad array of polar lipids includes species for which the polar head groups are neutral (cerebrosides), cationic (phosphatidylcholine) and anionic (sulfatides).

PHOSPHATIDYLCHOLINE



CEREBROSIDE, X=H

SULFATIDE, X=SO_3H



Scheme 1.

A number of publications have reported similarities between the various desorption techniques[8,9] and particularly their similarity with chemical ionization[10] in providing even-electron (generally protonated) molecular ion species, even while the mechanisms for primary particle (or photon) energy deposition, energy isomerization in the substrate and ion formation may be quite different.[8,11,12] On the other hand, there are significant differences between the ionization techniques which would suggest that the polar lipids from the mammalian membranes examined in this study would respond with different sensitivity to the various ionization/desorption techniques. While evidence has been offered to support both the desorption of *pre-formed* ions from solution[13] as well as gas-phase protonation of neutral species[14] in FAB, it is clear that the *charge sign* of ionic molecular species can be exploited in this technique.[10] In positive ion FAB mass spectra, molecules containing a quaternary nitrogen moiety are desorbed more easily than polar but neutral molecules,[15] and the solution pH (relative to the isoelectric point) effects the distribution of singly, doubly and triply charged molecular ions in the mass spectra of peptides.[16] Conversely, oligonucleotides containing anionic phosphate groups are more effectively analyzed using negative ion FAB mass spectrometry.[17] Similarly, PD has been used in the positive ion mode for very large peptides[18] and in the negative ion mode for oligonucleotides.[19] Laser desorption using a carbon dioxide laser (on the other hand) appears to be quite different. Both Kistemaker[20] and Cotter[21] have demonstrated that the primary mechanism involves the gas-phase cationization (alkali ion attachment) of desorbed neutral species. The effectiveness of the carbon dioxide laser for desorbing neutral species has resulted in a number of applications for the analysis of carbohydrates,[22,23] esterified phosphoglycolipids[24–27] and neutral polymers such as polyethylene and polypropylene glycols.[28,29]

Thus, in this study PD, LD and liquid SIMS were used to exploit their selective desorption of neutral, anionic and cationic biomarkers in and to provide complementary information for myelin membranes from rat brain and microsomes from rat liver. Mass spectra from the three techniques were all obtained on time-of-flight instruments. Intact myelin and microsomes were chosen for this study because they are both rich sources of well-characterized lipid mixtures, and because they are simple models for more complex cells and microorganisms. The total lipid content of myelin comprises about 80% of the dry weight of the membranes, and that of microsomes about 50% of the dry weight. In both cases membrane protein accounts for most of the remainder.

EXPERIMENTAL

Myelins and microsomes were prepared from 30-day old Sprague-Dawley rats (CD strain from Charles River Laboratories, Wilmington, Massachusetts). The animals were decapitated and brains and livers were removed and washed immediately with 0.9% NaCl. Myelins were isolated from the brain according to the method described by Norton.[30] Brains were homogenized in 0.32 M sucrose and layered over 0.85 M sucrose. After centrifugation, crude myelins were collected from the interface of the two sucrose solutions. After being washed and subjected twice to osmotic shock, myelins were isolated by the second discontinuous centrifugation as described above. The myelins were then collected from the interface, dialyzed and lyophilized.

Rat livers were immediately exsanguished in saline solution and microsomes were isolated as described by Narimatsu *et al.*[31] The perfused livers were homogenized in 0.32 M buffered sucrose (pH 7.4) and the homogenate was centrifuged stepwise at increasing gravity force. The microsomes were obtained between centrifugations at $10\,000 \times g$ for 20 min and $105\,000 \times g$ for 50 min. They were washed with 1.15% KCl, with distilled water and lyophilized.

Lyophilized myelins and microsomes were weighed and aliquots of approximately 10 μg or more were analyzed by mass spectrometry without further processing. Lyophilized hydroxycerebrosides, nonhydroxycerebrosides and sulfatides from calf brain were analyzed similarly.

LD, liquid SIMS and PD mass spectra were all obtained on time-of-flight instruments. LD mass spectra were obtained on a CVC 2000 (Rochester, New York) time-of-flight mass spectrometer, equipped with a Tachisto (Needham, Massachusetts) model 215G pulsed carbon dioxide laser. This configuration has been described earlier.[32,33] Liquid secondary ion mass spectra were obtained on a time-of-flight mass spectrometer designed and fabricated in our laboratory.[34,35] This instrument is equipped with a Kratos (Ramsey, New Jersey) Minibeam I rare gas ion gun and a Galileo (Sturbridge, Massachusetts) dual channelplate detector

floated at ±12 kV to provide post-acceleration of the secondary ions. Analog spectra are recorded and digitized by a LeCroy (Spring Valley, New York) 3500SA 100 MHz waveform recorder and signal averager. PD mass spectra were obtained on a BIO-ION Nordic (Uppsala, Sweden) model BIN 10K time-of-flight mass spectrometer using a 1 μCi ^{252}Cf source.

In each case, sample preparation and matrices were used which were most appropriate to the particular ionization technique. Thus, potassium chloride was added to the sample probe tip for LD to enhance formation of MK^+ ions; samples to be analyzed by liquid SIMS were dissolved in glycerol (positive ion) or diethanolamine (negative ion); and aqueous sample suspensions were evaporated on mylar-backed aluminum sample foils for PD. All spectra were transferred to an IBM PC-AT and baseline subtracted by in-house software to simplify comparison. This process removed the continuum of metastable (PD) or chemical (liquid SIMS, LD) noise which typically exists in desorption time-of-flight mass spectra.

RESULTS AND DISCUSSION

Positive ion mass spectra of myelin

The major lipid contents of rat brain myelin and rat liver microsomes have been studied by a number of investigators[36,37] and are summarized in Table 1, along with average molecular weights of dominant or representative members of each lipid family.

The positive ion mass spectrum of myelin obtained with the liquid SIMS time-of-flight mass spectrometer is shown in Fig. 1. The peaks at 735, 761 and 789 u are the protonated molecular ions for the heterogeneous phosphatidylcholines (PC) containing 16:0/16:0, 16:0/18:1 and 18:0/18:1 fatty acids, respectively. PC is unusual among the phospholipids in that loss of the polar head group is not observed in positive ion mass spectra, since the charge resides on the quaternary nitrogen. Instead, the polar head group is itself observed as the major ion at 184. Several other peaks are observed which may be attributed to losses of neutral fatty acids (RCOOH) or the acyl portions (RC=O) followed by H-transfer to the fragment ion, and are consistent with previous reports on the FAB mass spectra of PC.[38,39] For example, loss of the intact 16:0 fatty acid from *m/z* 735 or the 18:1 fatty acid from *m/z* 761 leads to the peak at 479. Loss of the acyl portion plus H-transfer from the same ions would lead to the peak 18 mass units higher at 497. Loss of the 16:0 fatty acid from *m/z* 761 might account for the peak at 505, etc. Loss of both fatty acids from all of the protonated molecular ions would remove the heterogeneity and result in the single peak at *m/z* 224. While there are a number of other peaks in the mass spectrum which cannot be easily accounted for, the liquid SIMS desorption of myelin shows high selectivity for PC and results in a highly interpretable mass spectrum.

This selectivity for the cationic PC component of myelin is more pronounced in the PD mass spectrum shown in Fig. 2, where there are fewer uninterpretable peaks, and where the mass spectrum closely corresponds to results obtained for pure PC.[40] The molecular ion abundance is greatly reduced relative to the formation of the highly stable quaternary phosphocholine head group fragment at *m/z* 184. The peaks at *m/z* 385 and 369 are the $[M-H]^+$ and $MH^+ - H_2O$ ions from cholesterol. The interpretation of the peak at 385 as a deprotonated cation of cholesterol in myelin was verified by obtaining the PD mass spectrum of pure cholesterol.

Table 1. Relative molarities of lipids in rat brain myelins and rat liver microsomes, and average molecular weights of selected species

	Relative myelin[a]	Molarities microsomes[b]	Average mol. wt	Ions observed
Cholesterol	100	20	386.6	+PD
PE	42	34	692.0, 718.0, 746.1[c]	
PC	25	100	734.1, 760.1, 788.1[c]	+PD, +liquid SIMS
Phosphatidylserine	15	7	736.0, 762.0, 790.1[c]	
PI	4	15	811.0, 837.1, 865.1[c]	
DPG	3	2	1353.9, 1379.9, 1408.0[d]	
Sphingomyelin	7	7	703.0, 731.1[e]	
Cerebrosides	33			
Non-hydroxy			728.1, 784.2, 812.3[f]	+LD
Hydroxy			744.1, 800.2, 828.3[f]	+LD
Sulfatides	7			
Non-hydroxy			808.2, 864.3, 892.4[f]	−PD, −liquid SIMS
Hydroxy			824.2, 880.3, 908.4[f]	−PD, −liquid SIMS
Ethanolamine plasmologen	33		674.0, 700.0, 728.0[g]	

[a] From ref. 44.
[b] From ref. 45.
[c] Calculated for the 32:0, 34:1 and 36:1 species.
[d] Calculated for the 64:0, 66:1 and 68:1 species.
[e] Calculated for the 16:0 and 18:0 species.
[f] Calculated for the 18:0, 22:0 and 24:0 species.
[g] Calculated for the 32:1, 34:2 and 36:2 species.

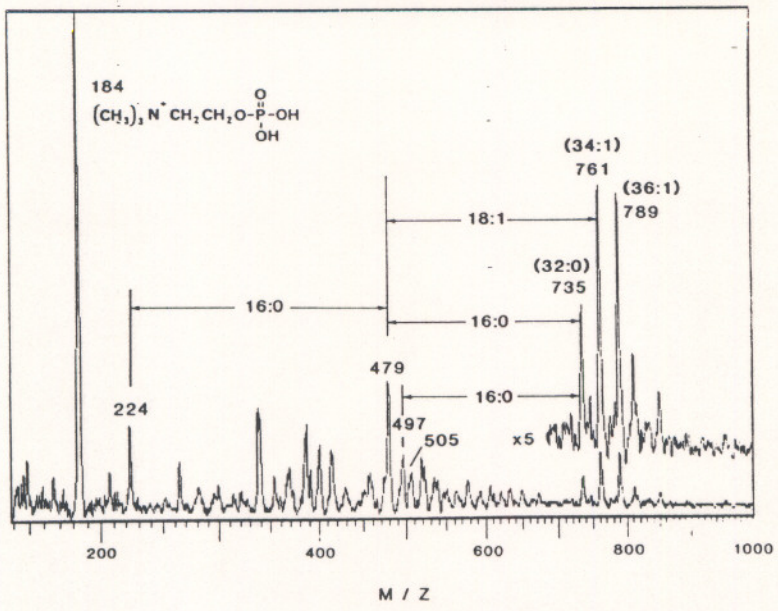


Figure 1. Positive ion liquid secondary ion mass spectrum of rat brain myelin.

In contrast, the formation of MK^+ ions of more neutral species in the LD technique accounts for the high selectivity for the cerebrosides and hydroxycerebrosides in myelin in the mass spectrum shown in Fig. 3(a). These are compared with the LD mass spectra of purified cerebrosides (Fig. 3(b)) and hydroxycerebrosides (Fig. 3(c)). Comparison of these spectra with one another and with the molecular weights listed in Table 1 verify that the peaks at 851, 823 and 767 are the MK^+ molecular ions for the non-hydroxy cerebrosides, while the peaks at 867, 839 and 783 arise from the hydroxycerebrosides. Thus, the observation of intact molecular ions for the neutral cerebrosides and hydroxycerebrosides provides complementary information to those methods (liquid SIMS and PD) which favor desorption of cationic species.

Negative ion mass spectra of myelin

Negative ion mass spectra provide selectivity for other classes of polar lipids, primarily the sulfatides. In the liquid secondary ion mass spectrum of myelin, shown in Fig. 4, the peaks at 823, 879 and 907 are the $[M - H]^-$ ions for the hydroxy sulfatides, while the peaks at 807, 863 and 891 are the molecular anions for the non-hydroxy sulfatides. Interestingly enough, there are a number of peaks which are anionic fragments of PC. Loss of $(CH_3)_3NH^+$ from PC molecules with molecular weights of 760 and 788 give rise to the peaks at 700 and 728, respectively. Loss of CH_3^+ from the same molecules results in the anions at 745 and 773. In both cases, the quaternary nitrogen cation is removed, giving a stable anion with the negative charge carried by the

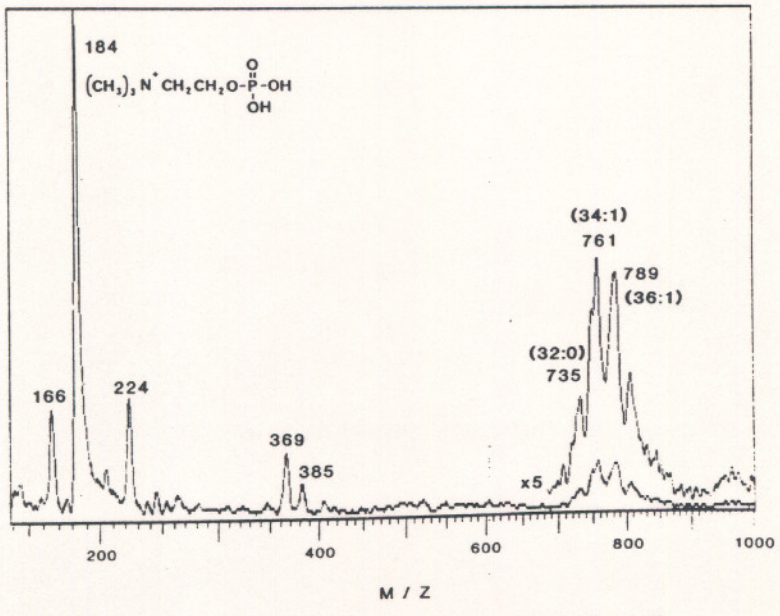


Figure 2. Positive ion PD mass spectrum of rat brain myelin.

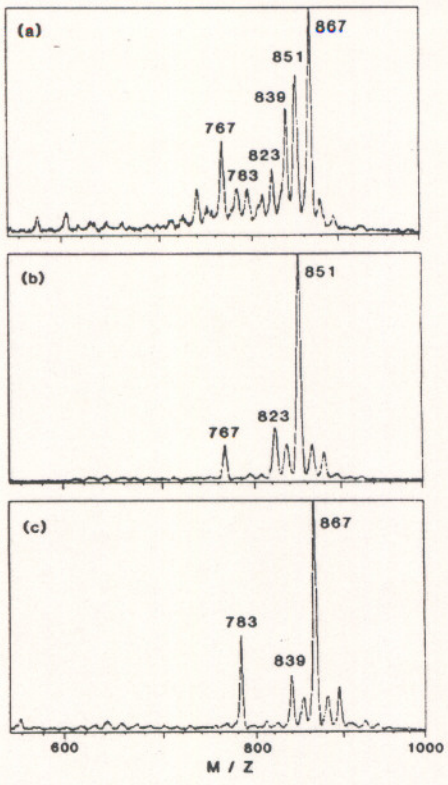


Figure 3. Positive ion LD mass spectra of (a) rat brain myelin, (b) calf brain non-hydroxycerebrosides and (c) calf brain hydroxycerebrosides.

phosphate group.[41] In addition, the peaks at 255 and 281 are the carboxylate anions for the 16:0 and 18:1 fatty acids, the predominant fatty acids in myelin phospholipids. Low-mass phosphate anions are observed in the liquid secondary ion mass spectra at 79 and 97, due to PO_3^- and $H_2PO_4^-$, respectively.

In the PD mass spectrum of myelin shown in Fig. 5, molecular anions of the hydroxy and non-hydroxy sulfatides are again observed, as well as the carboxylate anions at 255 and 281. In addition, the $[M - H]^-$ ion of cholesterol is also observed at 385.

The LD anion spectrum (Fig. 6) again shows a preference for neutral species. Sulfatide anions are not observed, while the $[M - H]^-$ peaks for the hydroxy cerebrosides at 743, 799 and 827 are observed. In addition, the carboxylate anions for the 16:0 and 18:1 fatty acids are observed at *m/z* 255 and 281, respectively.

Mass spectra of microsomes

Microsomes provide an interesting control for the experiments with myelins, since they contain the phospholipid families in proportions comparable to myelin (Table 1), but lack sulfatides and cerebrosides. In general, the response of the microsomal lipids under the three desorption techniques was consistent with the observations discussed for myelin. In the positive ion liquid secondary ion mass spectrum (Fig. 7), protonated molecular ions of phosphatidylcholine are observed at *m/z* 735, 761 and 789. In addition, losses of the 16:0 or 18:1 fatty acyl groups are observed as peaks at 497 and 523, while the polar head group again forms the base peak at 184.

While the PD technique again selects for the cationic PC, $[M + Na]^+$ ions are observed at *m/z* 757, 783 and 811 in the mass spectrum shown in Fig. 8, and may reflect an excess of sodium ions in this sample. The loss of neutral fatty acids and/or fatty acyl groups is not so readily apparent. However, the peaks at 369 and 385 again reveal the (somewhat smaller) presence of cholesterol as the $MH^+ - H_2O$ and $[M - H]^+$ ions, respectively. The phosphocholine head group is again the base peak.

No recognizable lipid molecular ions were observed in the LD mass spectra, perhaps reflecting the absence of glycolipids in microsomes.

In the absence of sulfatides, anionic fragment ions of PC dominate the high-mass region of the negative ion

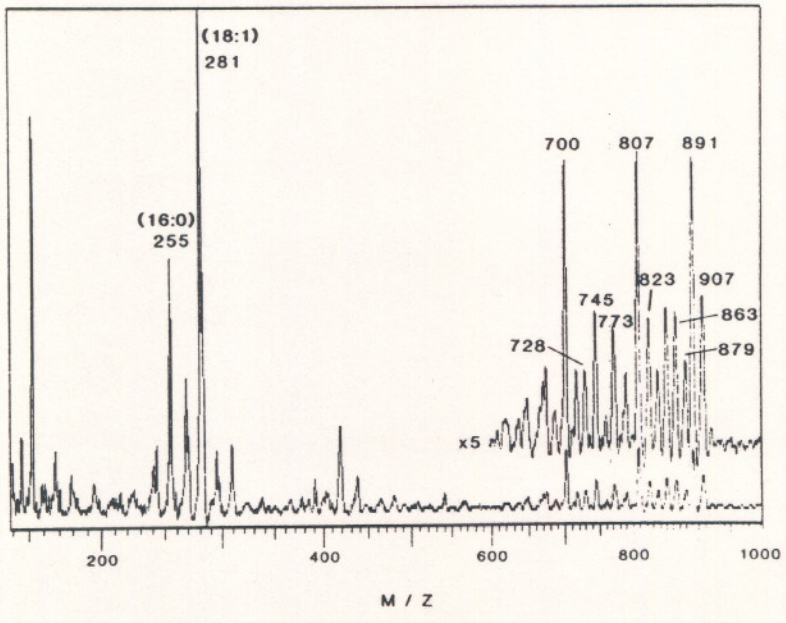


Figure 4. Negative ion liquid secondary ion mass spectrum of rat brain myelin.

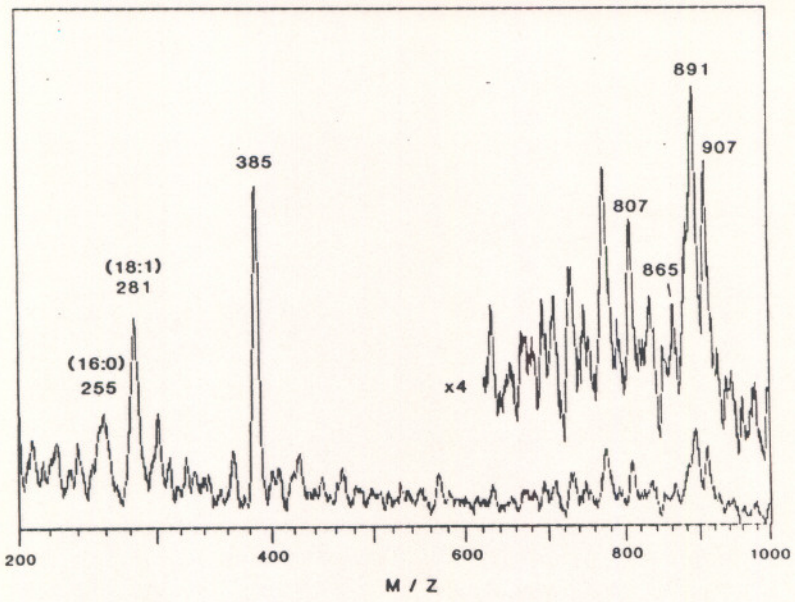


Figure 5. Negative ion PD mass spectrum of rat brain myelin.

liquid secondary ion mass spectrum of rat liver microsomes (Fig. 9). As before, loss of the $(CH_3)_3NH^+$ moiety from the neutral zwitterion leads to the anions recorded at *m/z* 674, 700 and 728. Loss of CH_3^+ produces the peaks at 745 and 733. The carboxylate anions for the 16:0 and 18:1 fatty acids are again observed at 255 and 281.

CONCLUSIONS

The selectivities for the lipid species which are detected by each of the techniques can be rationalized by features of the desorption processes suggested in the introduction. Desorption of *pre-ionized* involatile molecules of the appropriate charge sign is favored in PD and liquid SIMS or FAB.[15] Thus, PC are detected in cation spectra and sulfatides in anion spectra using these methods. LD volatilizes neutral species, i.e. cerebrosides, which are cationized in the gas phase. The mechanisms for formation of anions under LD are not yet defined, but will have to accommodate the present observations.

The desorption efficiencies of the other phospholipids using PD and liquid SIMS are apparently less than that of the choline-containing species when the complex mixture is analyzed. This has also been documented for lipids from amniotic fluid.[42] The inefficient desorption of pure phosphatidylserine by FAB has been reported.[39]

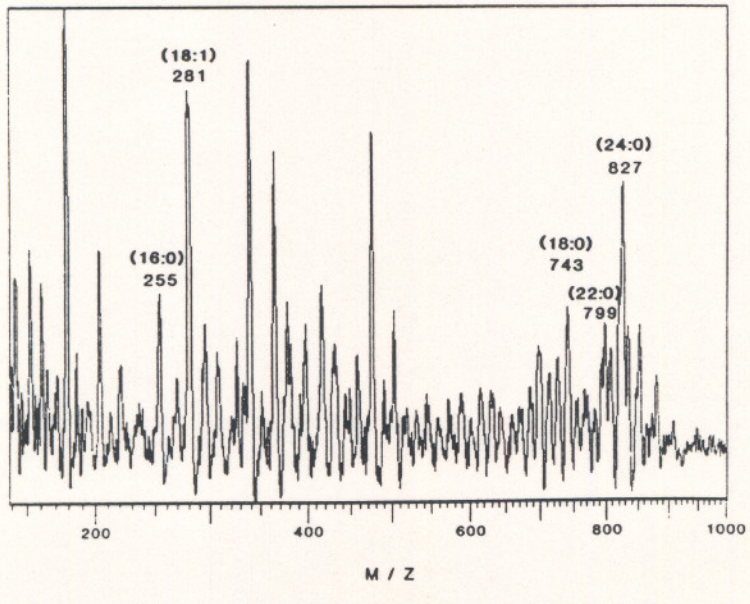


Figure 6. Negative ion LD mass spectrum of rate brain myelin.

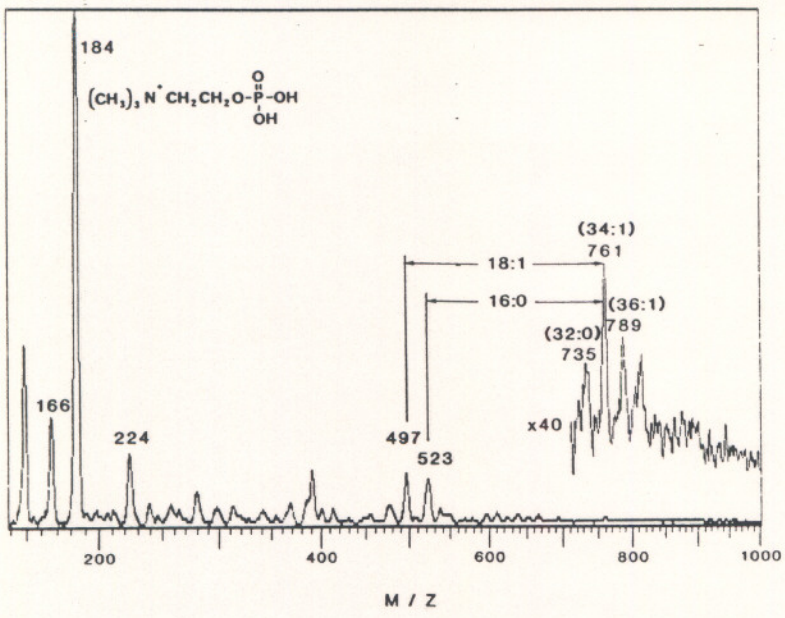


Figure 7. Positive ion liquid secondary ion mass spectrum of rat liver microsomes.

We have found that PE produces good PD or FAB mass spectra alone; however, in a 1:1 mixture with PC it is not detected by PD mass spectrometry and is strongly suppressed in FAB at higher concentrations. Presumably this preferential desorption reflects the presence of the positively charged quaternary ammonium center. Quaternary ammonium species have also been selectively desorbed from plants and plant extracts.[43] On the other hand, while several of the phospholipids are expected to exist as their phosphate anions, they are desorbed less readily than the anionic sulfatides. Thus, influential factors in addition to charge state remain to be characterized.

It should also be remarked that sensitivity for PC in PD appears to be comparable when they are desorbed either from myelin or from purified samples. The secondary ion signal for total PC species desorbed by PD mass spectrometry from 200 μg of myelin, where it constitutes 10% of the dry weight, is comparable to the ion signal from a 20 μg sample of the 16:0/16:0 homolog integrated through the same time.

In summary, different desorption techniques and different ion signs select for different lipid components of the complex mixtures that constitute myelin and microsome membranes. Characterization of membranes, cells and tissue may benefit from the application of several ionization techniques and awareness of their different selectivities.

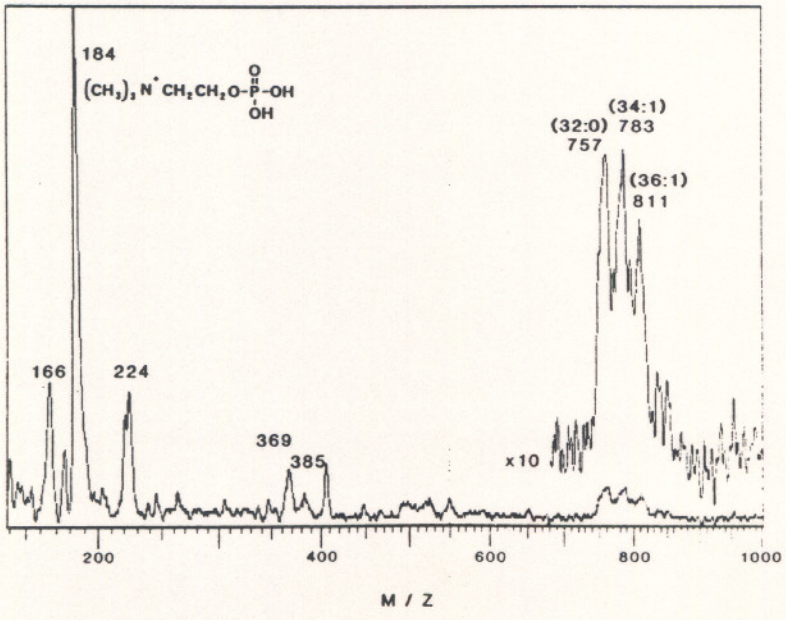


Figure 8. Positive PD mass spectrum of rat liver microsomes.

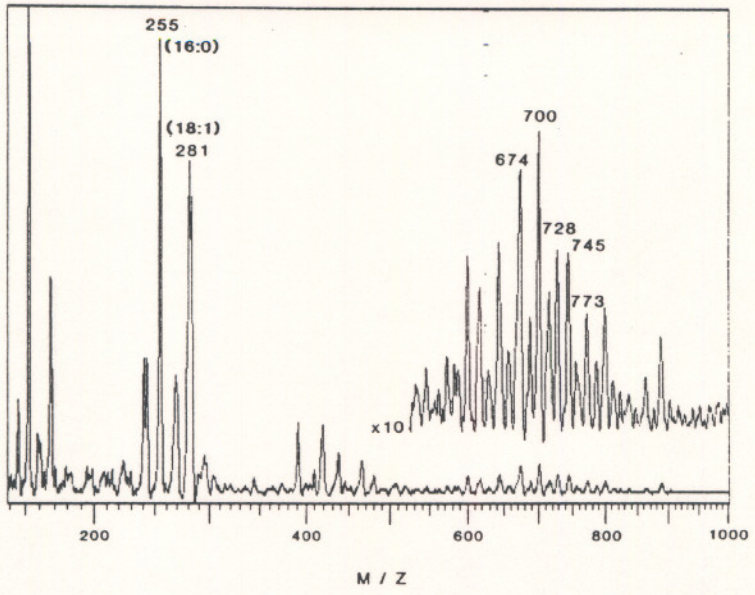


Figure 9. Negative ion liquid secondary ion mass spectrum of rat liver microsomes.

Acknowledgements

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