N-Alkylnicotinium Halides: A Class of Cationic Matrix Additives for Enhancing the Sensitivity in Negative Ion Fast-Atom Bombardment Mass Spectrometry of Polyanionic Analytes

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The addition of some surfactants to the fast-atom bombardment (FAB) matrix previously has been demonstrated to enhance analyte signals in fast-atom bombardment mass spectrometry. In particular, cationic surfactants appear to enhance the negative ion FAB detectability of analytes that exist as anionic species in the matrix solution. It has been proposed that the charged surfactant concentrates the oppositely charged analyte near the surface, which results in larger signals for the analyte. Cationic surfactants that contain a fixed positive charge and an additional basic site were prepared with different hydrophobic moieties and were evaluated for their effectiveness as FAB matrix additives. The compound N-octylnicotinium bromide (ONBr) is shown to improve greatly the analyte-related signals in negative ion fast-atom bombardment mass spectrometry for a variety of polyanionic analytes, relative to other surfactants (e.g., cetylpyridinium salts). This surfactant not only enhances detectability, but also simplifies the pseudomolecular ion region of the resulting spectra by reducing or eliminating metal cation adduct peaks. The simple mechanism of enhancement via surface activity is evaluated, and alternative mechanisms are considered. It is clearly shown that ONBr, as a FAB matrix additive, will allow mass spectrometry to be used for the analysis of anionic compounds that normally exhibit very low responses. (*J Am Soc Mass Spectrom 1994, 5, 935–948*)

Negative ion fast-atom bombardment mass spectrometry (NI-FAB-MS) has been the method of choice for the characterization of complex nucleotides [1] and other compounds that form anionic species in solution. Such analytes show better response in the negative ion mode compared to the positive ion mode, but the resulting mass spectra are relatively simple. Although this technique has proven to be somewhat effective for a large number of thermally labile, hydrophilic biomolecules such as nucleoside cyclic mono-, di-, and triphosphates [2–4], di-, tri-, and tetranucleotides [5–7], oligonucleotides [8–10], nucleotide sugars [11–13], cofactors [14], sugar phosphates, and sulfates [15–18], the use of NI-FAB-MS is frequently limited, particularly when the compounds form polyanionic species in solution. Sensitivity is generally low due to poor desorption and/or ionization efficiencies, so that rather large sample sizes (10 nmol or more) are required [2–18]. Although molecular weight information is provided, negative ion fast-atom bombardment (NI-FAB) mass spectra often exhibit few fragment ions; thus, the structural information available from the mass spectrum is limited. Moreover, interpretation can be complicated by the prominent clusters of ions generated because of adduct formation with metal cations such as Na^+. These adducts are usually unavoidable because alkali metal salts are present in the biological media and the buffers used to isolate and purify these compounds. Intense mass spectral peaks that represent matrix-related ions also can obscure analyte fragment ions in the low mass region of the spectrum.

A considerable amount of work has been reported concerning factors that may affect sensitivity in the analysis of polar compounds by fast-atom bombardment (FAB) and liquid secondary ion mass spectrometry (LSIMS) [19–23]. Although it is well established that positively charged species are best analyzed in the positive ion mode and negatively charged species are best examined in the negative ion mode, at least when the net charge in solution is 1, other factors obviously

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are very important as well [24, 25]. Ligon [26–29] suggested that, after charge state, another physical property that influences sensitivity is the surface activity of the analyte in the matrix. Those compounds that have high surface activity should be preferentially concentrated at the surface and should be detectable, even when present in low amounts. Analytes that lack surface activity may not be observed at all.

Enhancement of the surface concentration of an analyte can be achieved by two major strategies. One is to improve the surface activity by increasing the hydrophobicity of the analyte through lipophilic derivatization [30, 31]. In a second approach, the addition of appropriate surfactants that carry a charge opposite in sign to that of the analyte can enhance analyte surface concentration through attractive ionic interactions. A demonstration of the use of a cationic surfactant to improve signals in NI-FAB was reported by Ligon and Dorn [32] and later confirmed by Barofsky and co-workers [33]. It was found that the abundance of the $[M - H]^-$ ion for adenosine triphosphate (ATP) was increased by a factor of 10–50 (full scan data) by using cetylpyridinium salts (see Figure 1a) as matrix additives. Surfactant-induced signal enhancement also has been demonstrated in the context of continuous flow FAB (CF-FAB) [34] and reverse-phase high-performance liquid chromatography (HPLC) CF-FAB [35].

The previous work on matrix additives led Ligon to propose that the formation of an ion pair between the anionic analyte and the cationic surfactant plays a key role in sample enrichment at the surface, subsequently leading to signal enhancement, although no direct evidence for the analyte–surfactant complex was reported
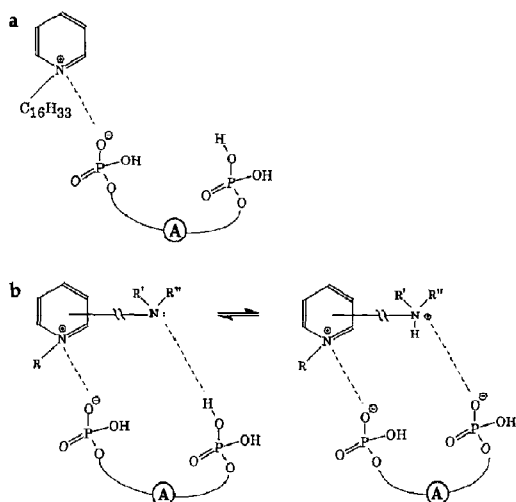
[26]. If ion-pair formation does indeed occur, as indicated in Figure 1a, then analogs of cetylpyridinium salts could be designed for higher sensitivity in the NI-FAB analysis of species that carry multiple negative charges in solution. A simple variation might be to utilize a surfactant with two permanent cationic sites. A more versatile molecule might contain one fixed cationic site and an additional basic group. If the basic group is an amine, then a number of ionic and hydrogen-bonding interactions could occur, depending on the sample and the solution pH, as suggested in Figure 1b. These "bifunctional" surfactants could act more effectively in the FAB matrix as ion-pairing reagents.

We have designed and synthesized a number of different types of such surfactants and have evaluated their utility as FAB additives. In this article we demonstrate that N-alkylnicotinium halides can be used as a class of highly efficient, cationic, bifunctional surfactants that significantly enhance the signals produced in the NI-FAB mass spectrometry of nucleotide analogs and other hydrophilic compounds that form polyanionic species in solution.

Experimental

Materials

Coenzyme A (CoA) sodium salt, all acyl coenzyme A's, coenzyme A glutathione disulfide (GS-SCoA) sodium salt, chondroitin disaccharide disulfate di-S_B trisodium salt, trigalacturonic acid, and uridine 5'-diphosphoglucuronic acid sodium salt were purchased from Sigma Chemical Company (St. Louis, MO). (S)-(-)-Nicotine, n-hexyl through n-dodecyl bromides, 2-(trimethylsilyl)ethoxymethyl chloride (SEMCl), pentafluorobenzyl bromide (PFBBr), 1,1,1,2,2,3,3,4,4,5,5,6,6-tridecafluoro-8-iodooctane, inositol hexaphosphate dodecasodium salt (myo-inositol hexakis[dihydrogen phosphate] dodecasodium salt, abbreviated $IP_6 \cdot Na_{12}$), ethylenediamine-tetraacetic acid (EDTA) disodium salt, and cetylpyridinium chloride (CPC) were obtained from Aldrich Chemical Company (Milwaukee, WI). Carboxyribose 1,5-diphosphate pentalithium salt was provided by Dr. M. Harpel, Biology Division, Oak Ridge National Laboratory (Oak Ridge, TN).

Preparation of N-Substituted S-(-)-Nicotinium Halides.

Quaternization by N-alkylation of the pyridine ring of nicotine was achieved by following established procedures [36]. The example used in this discussion will be the preparation of N-octylnicotinium bromide (ONBr). Prior to alkylation, the S-(-)-nicotine was purified by passing the liquid through a Pasteur pipet packed with 100-mesh silica gel. A mixture of 16.2 mg (0.10 mmol) of S-(-)-nicotine and 19.3 mg (0.10 mmol) of n-octyl bromide in 2 mL of methanol was heated in a tightly

Figure 1. (a) Proposed ion-pair formation between a phosphate-containing analyte and the cetylpyridinium cation. (A) represents the portion of an analyte molecule between two phosphate groups. (b) Proposed interaction between a multiply charged analyte and a bifunctional surfactant.

capped microvial at 110 °C for 4 h. Heating was discontinued when all of the *n*-octyl bromide had been consumed, as indicated by the disappearance of the $[M + H]^+$ peak of nicotine at m/z 163 in the positive ion FAB mass spectrum obtained by using glycerol as the matrix. Conversion was found to be quantitative. The resulting pale yellow solution was decolorized with activated charcoal. Without additional purification, this solution was used directly for the preparation of standard solutions in methanol–water (1:1 v/v).

Both positive ion mode and negative ion mode FAB-MS were used to verify the structure and purity of the final product. In the positive ion FAB (PI-FAB) spectrum, diagnostic peaks (and their relative intensities) for the successful preparation of ONBr include the octylnicotinium ion ON^+ at m/z 275 (100), and peaks that represent characteristic fragment ions observed at m/z 218 (6), 192 (10), 161(5), and 84 (16). The origin of these fragment ions was confirmed by a B/E scan for the ON^+ ion and by comparison with the fragmentation of several other analogs. In the NI-FAB mass spectrum with glycerol as the matrix, peaks that represent $[ON + 2Br]^-$ are observed when a large sample ($> 10\ \mu g$) of ONBr is used, which verifies the mass of the ON^+ ion and confirms that the counterion is in fact bromide.

Preparation of the other quaternary nicotinium salts required a heating period of 4–6 h to complete the reaction except for *N*-2-(trimethylsilyl)ethoxymethylnicotinium chloride, which was prepared by heating the reactants for 1 h at 110 °C. All products were checked for authenticity and purity by using PI-FAB-MS and NI-FAB-MS as described above.

Mass Spectrometry

Mass spectra were acquired on a JEOL (Peabody, MA) HX-110 double-focusing mass spectrometer (EB configuration) operating in the negative and positive ion modes. Ions were produced by bombardment with a beam of Xe atoms (6 keV). The accelerating voltage was 10 kV and the resolution was set at 1000. For collisionally activated dissociation–tandem mass spectrometry (CAD-MS/MS), helium was used as the collision gas in a cell located in the first field-free region. The helium pressure was adjusted to reduce the abundance of the precursor ion by 50%. A JEOL DA-5000 data system generated linked scans at a constant B/E. The instrument was scanned at a rate of 30–40 s from m/z 0–1500.

The JEOL DA-5000 data system reports the intensity of a peak as the area measured in arbitrary units. This value is proportional to the ion current. Enhancement of sensitivity was evaluated by comparing the intensities of the $[M - H]^-$ peaks in the spectra obtained by full mass scans in the absence and the presence of the surfactant additive. Peak intensities can vary significantly due to slight differences in the point at which data collection begins (refer to the section on opera-

tional considerations for a discussion of the time dependence of the analyte signal). Although every attempt was made to control this variable, its effects could not be eliminated completely. Because of the limitations of this experimental approach, the quantitative results cannot be taken as absolute; however, they do represent the overall trends observed in these studies and are useful for comparative purposes.

Typically, analyte solutions were prepared at a concentration of 0.5 $\mu g/\mu L$ in methanol–water (1:1 v/v). For FAB-MS analysis, 1 μL of a mixture of glycerol–MeOH–H$_2$O (2:1:1 v/v/v) was applied to the probe tip followed by 1 μL of the analyte solution, and finally 1 μL of the surfactant solution (1 $\mu g/\mu L$). To optimize conditions for an analyte, sample sizes between 0.5 and 1 nmol should be used. The best results are obtained when the amount of surfactant added to the FAB sample is evaluated over a range of 0.1–5.0 μg (0.3–14 nmol for ONBr). When the surfactant is present in excess, a prominent triplet in the spectrum that represents $[ON + 2Br]^-$ ions at m/z 433, 435, and 437 will be observed. Rapid scanning (at least 30–40 s to scan from 0–1500 u) is recommended, with a minimal delay between the initiation of fast-atom bombardment and data collection.

Nomenclature and Conventions

All analytes were purchased as alkali metal salts. Consider a "polyanionic" species available as the hexasodium salt, which we will designate as ANa$_6$. In the experiments presented, the free acid form of this analyte, AH$_6$, will be used as a point of reference for mass, and will simply be denoted as M. Thus, a pseudomolecular ion may appear in the NI-FAB mass spectrum formed by deprotonation of M and labeled as $[M - H]^-$. The convention of labeling ions based on their free acid form rather than the actual salt that was introduced into the FAB experiment greatly simplifies the designation of the ionic species observed in this study. If the usual practice was followed where M = ANa$_6$, then the cited pseudomolecular ion would have the cumbersome designation of $[M - 6Na + 5H]^-$. In addition, a range of pseudomolecular ions is observed that contains various numbers of alkali ions, such as $[M - 5H + 4Na]^-$ and $[M - 4H + 3Na]^-$. For simplicity, these partially sodiated pseudomolecular ions will be referred to as adduct ions.

Results and Discussion

Preliminary Studies

A series of *N*-alkyl- and *N*-arylalkylnicotinium halides was synthesized, and their utility as FAB additives was evaluated. Their designations are indicated in Table 1. Figure 2 compares the ability of the bifunctional alkylnicotinium surfactants to alter the desorption–ionization efficiency of a model compound, coen-

Table 1. Structure and designation of N-alkyl- and
N-arylalkylnicotinium halides

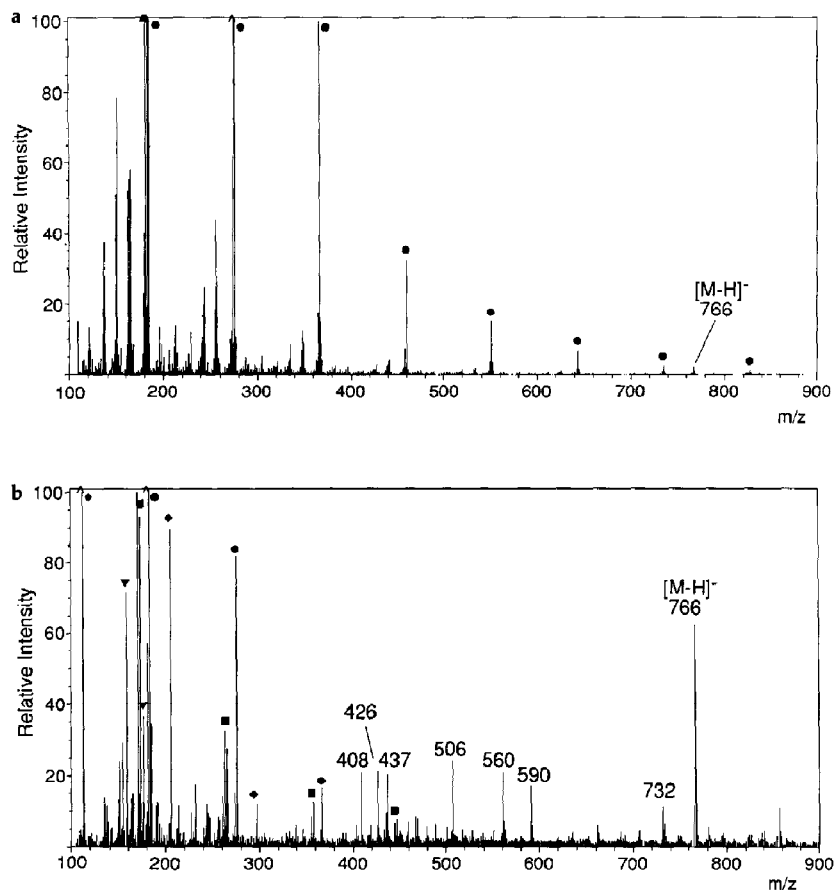|	Counterion	Designation
$R = n\text{-}C_6H_{13}$	$X = Br$	HxNBr
$R = n\text{-}C_7H_{15}$	$X = Br$	HpNBr
$R = n\text{-}C_8H_{17}$	$X = Br$	ONBr
$R = n\text{-}C_{10}H_{21}$	$X = Br$	DNBr
$R = n\text{-}C_{12}H_{25}$	$X = Br$	LNBr
$R = CF_3(CF_2)_5(CH_2)_2$	$X = I$	FONI
$R = C_6F_5CH_2$	$X = Br$	PFBNBr
$R = (CH_3)_3SiCH_2CH_2OCH_2$	$X = Cl$	SEMNCl

zyme A, and shows data from mass spectra obtained by repetitive scanning. Within the homologous series of N-alkylnicotinium halides that have a chain length between C_6 and C_{12}, the greatest signal enhancement for the $[M - H]^-$ ion of coenzyme A was achieved with N-octylnicotinium bromide (ONBr). Figure 2 also shows the time dependence of the signal intensity. The abundance of the $[M - H]^-$ ion from three scans (40 s/scan) was considered so that the maximum responses and the rates of signal decay could be compared. Though the maximum intensity of the analyte signal is relatively short lived when ONBr is present, the overall performance was found to be superior to the other alkylnicotinium compounds, even after signal decay. Derivatives containing n-hexyl and n-lauryl chains appear to be approximately as effective in assisting the desorption–ionization of coenzyme A as cetylpyridinium chloride (CPC). It is also apparent from Figure 2 that n-heptyl-, n-octyl-, and n-decylnicotinium bromides are markedly more efficient than CPC in enhancing the $[M - H]^-$ signal. Two fluorinated compounds (FONI and PFBNBr) and one
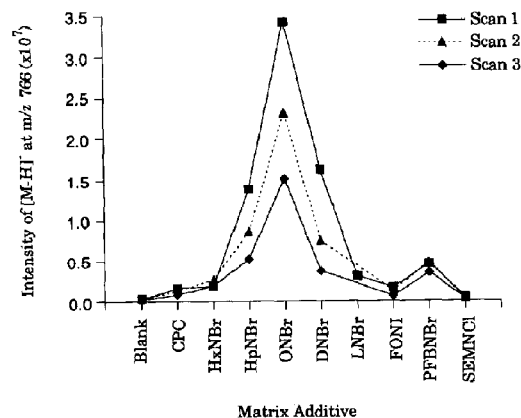
trimethylsilyl-containing analog (SEMNCl) contain highly lipophilic moieties and are potentially powerful surfactants (Table 1). It was found, however, that none of these compounds could provide signal enhancement greater than that produced by the simple alkyl-substituted quaternary nicotinium salts.

Figure 3 shows the NI-FAB mass spectra obtained from 0.5 µg (0.6 nmol) of coenzyme A sodium salt with and without the addition of surfactant. In the conventional FAB mass spectrum (Figure 3a), the intensity of the $[M - H]^-$ ion at m/z 766 is extremely low. Fragment ions are not observable above the level of the noise, and the spectrum is dominated by intense peaks generated from the glycerol matrix. However, the sample containing surfactant showed a remarkable increase in the intensity of the $[M - H]^-$ ion at m/z 766 (Figure 3b). Not only was the abundance of the $[M - H]^-$ ion species enhanced in these experiments, but prominent fragment ions appeared throughout the spectrum (Figure 3b). A literal comparison of the relative intensities for the peaks at m/z 766 might suggest that the peak in Figure 3b is approximately 20 times larger than the peak in Figure 3a. However, the relative intensities in the two spectra are not directly comparable because the data system normalized each one to a different peak with a different raw intensity value. Raw intensities for the same mass-to-charge ratio value can be compared because they are based on peak areas calculated in arbitrary units and are proportional to the ion current detected. By using this approach, the ion current for m/z 766 is increased by a factor of 37 upon addition of ONBr. The production of larger analyte ion currents made it possible to perform CAD-MS/MS on the $[M - H]^-$ ion and other precursors. The product ions generated from $[M - H]^-$ at m/z 766 by CAD-MS/MS (Figure 4) together with the fragment ions from the surfactant-assisted spectrum in Figure 3b provide a clear understanding of the fragmentation of coenzyme A (Figure 4) that is consistent with data reported in the literature for S-acyl derivatives of coenzyme A [37–39].

Although ONBr enhances the detectability of polyanionic analytes in NI-FAB, it also greatly simplifies the molecular ion region of the spectrum. This is both an important advantage in the analysis of many polyanionic species and an aspect that must be considered when establishing the mechanism through which ONBr operates. Figure 5a shows the molecular ion region of the NI-FAB mass spectrum of the pentalithium salt of carboxyribose 1,5-diphosphate. Not only are the signal intensities low, but the various alkali ion adducts spread the molecular weight information among many peaks, which distributes the available signal and raises the detection limits. When ONBr is added, this portion of the spectrum radically changes (Figure 5b). The peak that represents the $[M - H]^-$ form of the analyte clearly dominates, which suggests that ONBr not only enhances detectability but changes the chemical environment from which analyte desorption–ionization occurs.


Figure 2. Response of 0.5 µg of coenzyme A sodium salt in the presence of 1 µg of various N-substituted nicotinium halides (see Table 1 for designations). The response was evaluated on the basis of the intensity of the $[M - H]^-$ peak at m/z 766.

Figure 3. The NI-FAB mass spectra obtained from 0.5 μg of coenzyme A sodium salt: (a) without surfactant and (b) with the addition of 1 μg of ONBr. The intensity of the [M $-$ H]$^-$ ion at m/z 766 was 0.69 $\times$ 10^6 without ONBr and 25.51 $\times$ 10^6 with ONBr (factor of 37 increase). Refer to the discussion of mass spectrometry in the Experimental section for a description of enhancement factor calculations. Glycerol (G) ions are denoted by ●, bromide adduct ions [Br + nG]$^-$ by ■, and sodium adduct ions [nG $-$ 2H + Na]$^-$ by ◆. The intense peaks at m/z 177 and 159 represent the [$H_3P_2O_7$]$^-$ and the [$H_3P_2O_7$ $-$ H_2O]$^-$ ions, respectively, and are marked by ▼.

A third characteristic of ONBr as a matrix additive is its nearly "transparent" nature in NI-FAB-MS. The fixed positive charge on the *N*-octylnicotinium ion precludes its detection under NI-FAB-MS, although the bromide counterion is frequently observed in the low mass region of the spectrum alone and as an adduct to matrix molecules but not to the anionic analyte. In light of these desirable features, the range of applicability of ONBr was investigated in an extended study. Examples that follow show the extent to which this matrix additive simplifies spectra as it enhances detectability.

Application to Complex Biomolecules

One important class of anionic biomolecules that is difficult to analyze by NI-FAB-MS is coenzyme A analogs that contain highly polar substituents linked to

the thiol group. Coenzyme A glutathione disulfide (GS-SCoA) is particularly refractory to FAB-MS analysis. At least 10 μg of GS-SCoA was required to observe the [M $-$ H]$^-$ ion at m/z 1071 and a few fragment ions (Figure 6a), but adducts formed with as many as four sodium ions were prominent when this quantity of compound was analyzed. The adduct ions each lose the glutathione moiety so that the pattern of multiple sodium adducts was again observed in the lower mass region of the spectrum. Other fragment ions were formed but at much lower abundances. The addition of ONBr to the FAB sample resulted in an enhancement of the [M $-$ H]$^-$ peak intensity by nearly 3 orders of magnitude (Figure 6b). Moreover, the multiple sodium ion adducts were virtually eliminated. Intense peaks that represent fragment ions are also enhanced and appear throughout the spectrum, which allows the structural features of the coenzyme A portion of the molecule to be determined readily (Figure
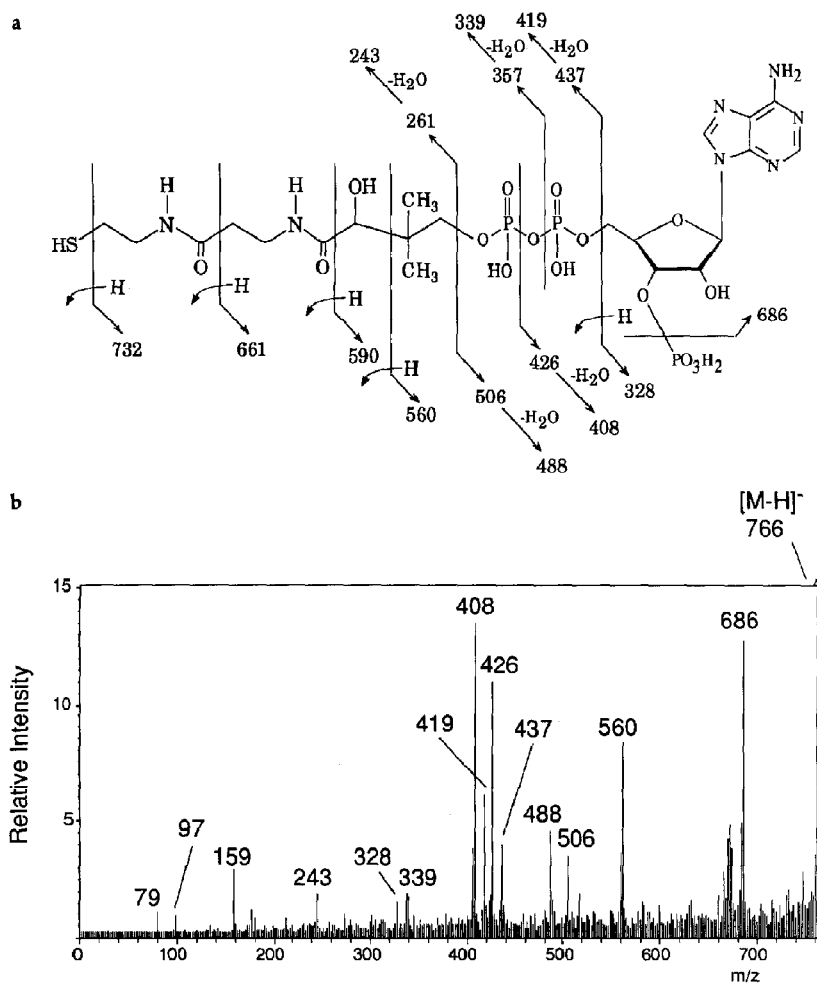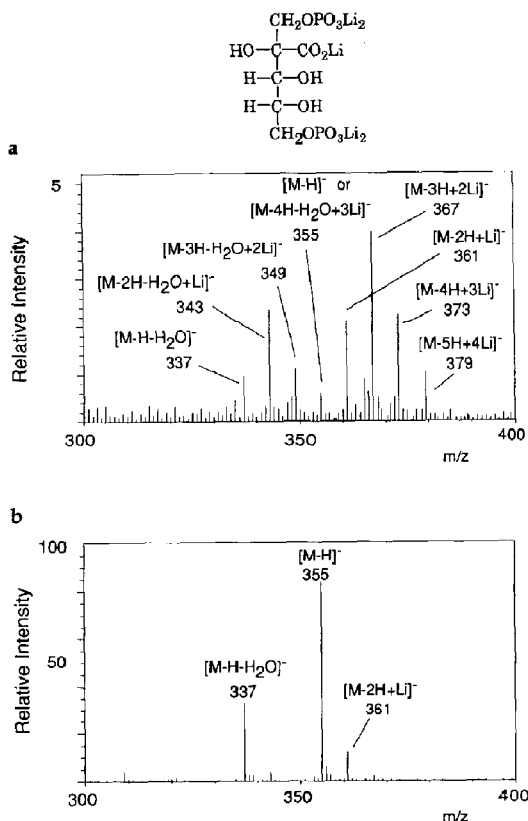
Figure 4. (a) Proposed fragmentation of coenzyme A and coenzyme A derivatives in NI-FAB-MS. Hydrogen shifts that accompany skeletal bond cleavages are also indicated. If the observed fragment is 1 mass unit lower than the value calculated for that portion of the molecule, then it contains the original site of deprotonation. (b) CAD-MS/MS spectrum of 0.5 μg of coenzyme A obtained by a B/E linked scan of the [M − H]⁻ precursor ion at m/z 766.

4a). The size of the substituent linked to the thiol group can be identified by the loss of glutathione to form the ion at m/z 766 and the ion formed by cleavage with charge retention on the tripeptide (γ-Glu-Cys-Gly) observed at m/z 306. Without ONBr, some of these structurally informative fragments cannot be observed, even at higher sample concentrations.

In addition to complicating the molecular ion region, salt contamination can sometimes completely suppress desorption–ionization of the analyte in FAB-MS [40]. Various desalting procedures have been suggested for sample cleanup prior to analysis: these include HPLC purification, solid-phase extraction, the addition of crown ethers [41], ion exchange [42–44], and sample washing on the FAB probe tip [45]. Another recommendation is the addition of acids to the

matrix to facilitate the exchange of metal ions for protons and the desorption of the analyte as singly charged ions [2, 46]. Even with these improvements, the required sample size remains quite large for the salts of polyanionic species [47]. We have found that surfactant-assisted FAB is an alternative procedure that can be used to eliminate interference from alkali cation contamination.

The effectiveness of ONBr for the elimination of cation adduct formation for clusters that contain a relatively small number of metal ions was demonstrated with carboxyribose 1,5-diphosphate penta-lithium salt (Figure 5), but its efficiency at the extreme is illustrated best with inositol hexaphosphate dodeca-sodium salt. Previously reported analyses with PI- and NI-FAB-MS were accomplished only by taking unusu-

$$CH_2OPO_3Li_2$$
$$HO-\overset{|}{\underset{|}{C}}-CO_2Li$$
$$H-\overset{|}{\underset{|}{C}}-OH$$
$$H-\overset{|}{\underset{|}{C}}-OH$$
$$CH_2OPO_3Li_2$$

a

b

Figure 5. NI-FAB mass spectra of 0.5 μg carboxyribose 1,5-diphosphate pentalithium salt (a) without surfactant and (b) with 1 μg ONBr. Note that the ion at m/z 355 detected without surfactant could also correspond to $[M - 4H - H_2O + 3Li]^-$.

ally large amounts of sample, in some cases as much as 200 nmol of the compound [15]. When a 100-μg sample of the dodecasodium salt of inositol hexaphosphate (which corresponds to 108 nmol of the free acid) was examined in our laboratory by NI-FAB-MS, the $[M - H]^-$ ion at m/z 659 was not detected. The high mass region of the spectrum was dominated by multiple sodium adducts of a fragment called IP_3, which is analogous to the structure shown in Figure 7a except three of the R groups have been replaced by hydrogen atoms and the remaining six sodium atoms also are replaced by hydrogen atoms. A radical change in the appearance of the spectrum occurred when ONBr was added to the FAB sample mixture (Figure 7b). Sodium ion adducts were much less abundant and the $[M - H]^-$ ion at m/z 659 became the dominant high mass species. Even the addition of NaCl to the FAB sample did not alter the efficiency of ONBr. An easily interpretable spectrum can be acquired with as little as 0.5 μg (0.54 nmol) of inositol hexaphosphate dodecasodium salt when ONBr is present. Loss of one phosphate group as H_2PO_3 and two others as H_3PO_4 results in the ions observed at m/z 579, 481, and 383.

It is interesting to note that the analyte–surfactant complex, $[M - 2H + ON]^-$, was detected at m/z 933. One of the major limitations to the analysis of inositol phosphates by conventional FAB-MS/MS techniques is the marked reduction in sensitivity with increasing phosphorylation [48]. The enhanced sensitivity that results from addition of ONBr to the FAB sample, together with the structural information obtained from regular loss of phosphate groups, suggest a potential application of our methodology to labeling studies of these intracellular second messengers.

Limited sensitivity also has been a problem in the analysis of organic sulfates and sugar sulfates by NI-FAB-MS [16–18]. We have found that the response of these compounds can be enhanced by ONBr as well. For example, chondroitin disaccharide disulfate (di-S_B) trisodium salt showed a thirty to forty fold increase in the intensity of the $[M - H]^-$ ion when surfactant was added to the FAB sample. Diagnostic fragment ions that were observed were consistent with those reported in the literature [16] and were sufficiently prominent to confirm the location of the sulfate group on each sugar moiety.

Molecules that contain carboxyl groups were also investigated. The NI-FAB-MS spectra of trigalacturonic acid obtained with and without surfactant were compared. Although the $[M - H]^-$ ion at m/z 545 is observable by conventional FAB-MS [49, 50], the signal is enhanced considerably with the addition of ONBr, and fragment ions were detected in the spectrum. These results suggest that the surfactant methodology also could be applied to the analysis of oligosaccharides that contain acidic functionalities.

Over the past year, ONBr has been used successfully in this laboratory to improve the NI-FAB-MS signals of over 80 structurally distinct compounds. In general, molecules that contain one or more phosphate groups respond most favorably. Some sulfates and carboxylates show a definite improvement in response, but the enhancement is smaller and often structure specific. Polyanionic species that possess lipophilic moieties like gangliosides exhibit no signal enhancement in the presence of ONBr. The largest enhancements are achieved for analytes with relatively low surface activity in the matrix. Some of the results of this extended study are presented in Table 2.

Operational Considerations

To optimize sensitivity in the analysis of polyanionic compounds by NI-FAB-MS in the presence of ONBr, we investigated several operational parameters; in particular the choice of FAB matrix, the order of addition of the solutions to the probe tip, the temporal aspects of data acquisition, and the ratio of analyte to surfactant. The results of these studies are summarized here to assist in the incorporation of ONBr into the FAB technique.
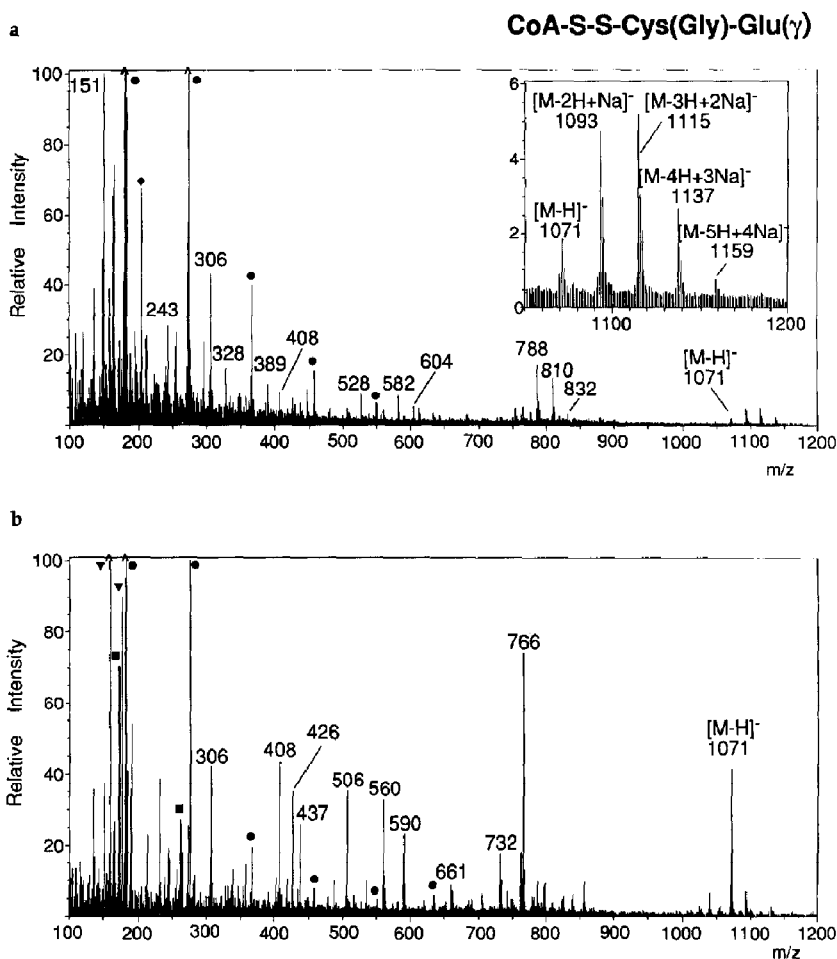
Figure 6. NI-FAB mass spectra of glutathione coenzyme A sodium salt: (a) 10 μg analyte, without surfactant, including an expanded view of the molecular ion region and (b) 0.5 μg analyte with 1.5 μg ONBr. The intensity of the $[M - H]^-$ peak at m/z 1071 was 0.38×10^6 without ONBr and 13.8×10^6 with ONBr (a factor of 726 increase, taking into account the difference in sample size).

In a FAB experiment, the matrix often can be changed to produce small improvements in analyte response. The typical basic matrices used in negative ion FAB-MS, such as triethanolamine, diethanolamine, and 3-aminopropanediol, were tested with the polyanionic analytes examined in these studies but failed to produce better signals than the signal generated with glycerol. When these matrices were combined with ONBr, little change in the analyte signal was observed. In fact, signal levels actually declined when ONBr was present. The maximum enhancement was achieved when glycerol was used as the matrix. Related analogs with lower volatility, such as tetraethylene glycol and diglycerol, increased signal lifetime but the enhancement produced when ONBr was added was not as great as that obtained with glycerol. For all other studies, we chose glycerol as the matrix but reduced the amount by using a mixture of glycerol–

MeOH–H₂O (2:1:1 v/v/v). Use of this solution permitted a reproducible quantity of glycerol to be applied to the probe tip with a syringe.

In most cases, the analyte signal intensity was found to change with time. Figure 8 shows the abundance of the $[M - H]^-$ ion at m/z 808, as a function of time, for a sample that contains 0.5 μg acetyl CoA lithium salt, 1.0-μg ONBr and 1 μL of the glycerol matrix solution. A control sample that did not contain surfactant is also included. In both cases, the largest ion currents were detected a short time after bombardment was initiated. Relative to the control, the signal level in the sample that contained surfactant declined rapidly early in the experiment, then decreased gradually with time. Therefore the point at which data acquisition begins will greatly affect the maximum ion currents recorded. In our work, spectra were routinely collected before the response decayed significantly.
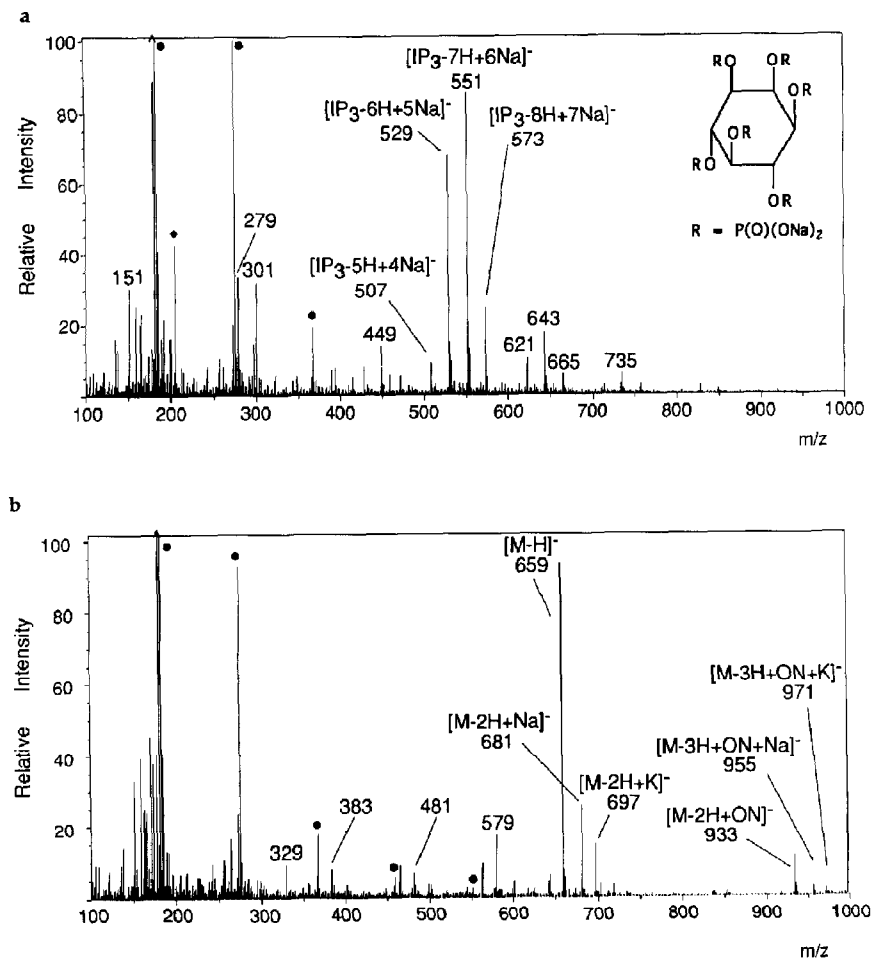
Figure 7. NI-FAB mass spectra of inositol hexaphosphate dodecasodium salt: (a) 100 μg analyte, without surfactant and (b) 0.5-μg analyte with 1.0-μg ONBr. Without ONBr, the [M − H]⁻ ion at m/z 659 was not observed above the level of the noise. The peaks observed at m/z 621, 643, 665, and 735 represent glycerol adducts to various sodiated IP_3 species. With ONBr, the intensity of the [M − H]⁻ ion was 26.3 × 10⁶. IP_6 represents the free acid composed of the inositol ring and six phosphate groups. IP_3 (free acid form) is the inositol ring that contains three phosphate groups and three hydroxyl groups.

The time dependence was found to vary somewhat for different analytes. Usually, the signal generated by surfactant-assisted FAB lasted for 3–5 min, which was sufficient to allow for full spectra and tandem mass spectrometry studies to be completed.

With the matrix and scanning parameters described above, we examined the effect of varying the ratio of surfactant to analyte. Figure 9 demonstrates the dependence of the relative abundance of the [M − H]⁻ ion obtained from 0.5 μg (0.65 nmol) of acetyl CoA lithium salt as a function of the amount of ONBr present. With a fixed amount of analyte, it is apparent that ONBr promotes signal enhancement over a range of quantities from 0.1 to 5.0 μg; the maximum effect occurs in the presence of 1.0 μg (2.8 nmol) of surfactant, which

corresponds to an ONBr-analyte mole ratio of about 4:1. A decline in the intensity of the [M − H]⁻ ion is observed with larger quantities of ONBr. The mass spectrum taken in the presence of 1.0 μg of the surfactant exhibited an intense [M − H]⁻ ion at m/z 808 together with peaks that represent structurally informative fragment ions. Stoichiometric ratios for other analytes were studied, and in each case the optimal value was found to fall between 4:1 and 6:1.

Analyte signal intensity as a function of the quantity of analyte in the presence of a fixed amount of ONBr was also investigated. Figure 10 shows the change in the response for the [M − H]⁻ ion at m/z 808, the [M − 2H + Li]⁻ ion at m/z 814, and the [M − 3H + 2Li]⁻ ion at m/z 820 with increasing

Table 2.   Examples of signal enhancement in NI-FAB-MS in the presence of ONBr[a]

Analyte	Enhancement factor of $[M - H]^-$ in full scan	Molecular weight of the free acid (nominal mass)
Nucleotides		
Coenzyme A, sodium salt	37	767
Acetyl coenzyme A, lithium salt	16	809
Linolenoyl coenzyme A	8	1029
Malonyl coenzyme A, lithium salt	93	853
Coenzyme A glutathione disulfide, sodium salt	726	1072
Flavine adenine dinucleotide, disodium salt	37	785
Vitamin B_{12}	12	1355
Adenosine triphosphate, sodium salt	27	507
Uridine 5'-diphosphoglucose, sodium salt	18	566
Sugar phosphates		
Carboxyribose 1,5-diphosphate, pentalithium salt	573	356
Inositol hexaphosphate	68	660
Inositol hexaphosphate, dodecasodium salt	b	660
Phospho(enol)pyruvate, trisodium salt	13	168
Sulfates		
Chondroitin disaccharide sulfate 6-S, disodium salt	26	459
Chondroitin disaccharide disulfate di-S_B, trisodium salt	36	539
17β-Estradiol 3-glucuronide 17-sulfate, dipotassium salt	7	528
Carboxylates		
Trigalacturonic acid	10	546
Sialyllactose	12	633
Benzylpenicillin, sodium salt	4	334
EDTA, disodium salt	30	292

[a] All samples contained 0.5 μg analyte and 1–2 μg ONBr. Enhancement factors represent the ratios of peak areas for single scans.
[b] An enhancement factor could not be calculated because the $[M - H]^-$ ion was not observed without surfactant (see Figure 7).

amounts of acetyl CoA lithium salt when 1.0 μg (2.8 nmol) of ONBr was added to the FAB sample. This quantity of surfactant was effective in enhancing the signal of acetyl CoA in the range from 0.05 to 5.0 μg (65 pmol to 6.5 nmol). The maximum enhancement for the $[M - H]^-$ ion was observed with 0.5 μg (0.65

nmol) of analyte. Thus for a given amount of analyte in the presence of insufficient quantities of surfactant, the alkali ion adduct peaks will predominate (Figure 10). When larger quantities of analyte (e.g., 5 μg) are used, the $[M - H]^-$ ion again will dominate the spec-
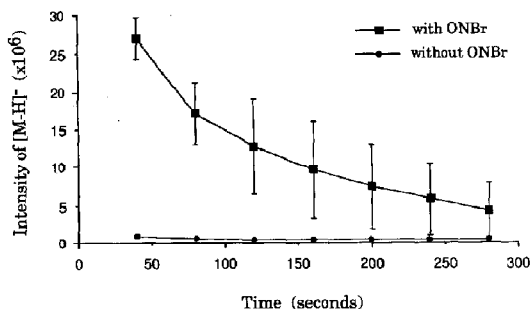
Figure 8.   Intensity of the $[M - H]^-$ peak at m/z 808 as a function of fast-atom bombardment time for 0.5 μg of acetyl CoA lithium salt with and without 1 μg of ONBr. Each point represents the mean of three samples. Error bars for the curve without ONBr are smaller than the size of the symbol.
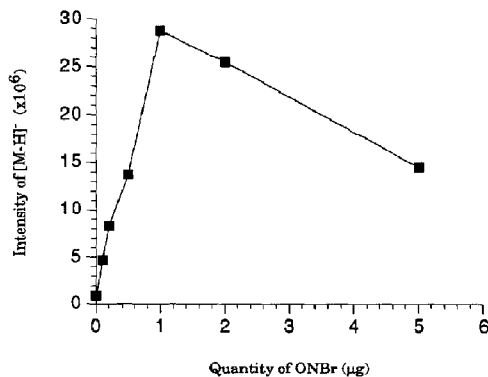
Figure 9.   Intensity of the $[M - H]^-$ peak at m/z 808 for 0.5 μg acetyl CoA lithium salt as a function of the quantity of ONBr added to the sample. Each point represents the sum of the first three scans collected from a single sample.
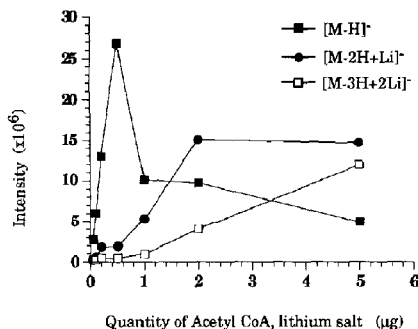
Figure 10. The intensities of the $[M - H]^-$ ion at m/z 808, the $[M - 2H + Li]^-$ ion at m/z 814, and the $[M - 3H + 2Li]^-$ ion at m/z 820 as a function of the amount of acetyl CoA lithium salt in the presence of a fixed amount of ONBr (1.0 μg). Each point represents the mean of the first three scans collected from a single sample.

trum only if more ONBr is added. Any further increase in the amount of analyte resulted in a gradual decrease in the intensity of the signal for the $[M - H]^-$ ion accompanied by an increase in ions that represent the alkali ion adducts. Although an analyte–surfactant mole ratio of 1:5 is adequate for low microgram quantities of CoA and its analogs, the same stoichiometric ratio may not be optimal for other classes of compounds. Furthermore, when much smaller quantities of the analyte are used, it may be necessary to increase the amount of ONBr added to the FAB sample. For example, 10 ng of acetyl CoA required 100 ng of ONBr to achieve a signal-to-noise ratio of 7:1.

Mechanistic Considerations

In all of the experiments reported here that involve the addition of ONBr to the FAB matrix for analysis of polyanionic compounds, two distinct phenomena repeatedly were observed. First, the response in NI-FAB was significantly enhanced in the presence of surfactant, particularly the intensity of the $[M - H]^-$ ion. Second, the composition of the molecular ion region changed drastically in the spectra obtained from the alkali salts of analytes.

Different approaches may be used to explain how these observations could result from a change in the FAB sample when ONBr is added to the mixture. The simplest mechanism is based on enhanced desorption and has already been proposed. As a cationic surfactant, ON^+ resides on the surface of the glycerol. The anionic analyte accumulates at or near the surface via ion-pair interactions with ON^+. Thus, the analyte concentration is enhanced at the matrix surface, which leads to the sampling of a larger number of analyte ions. This is consistent with the fact that the analyte signal is often not as long lived when surfactant is present compared to analyses performed without surfactant in which the glycerol–analyte mixture is more homogeneous. If ON^+ forms complexes with the

polyanionic analyte, then these analyte–surfactant interactions are stronger than the analyte–metal cation interactions. Alkali cations then would be displaced from anionic sites in the analyte. The chemical environment from which the analyte desorbs as a singly charged species is depleted of alkali cations and enriched with ON^+. This new chemical environment can provide protons to anionic sites to reduce the net charge. Thus, the change in composition of the pseudomolecular ions formed from the analyte demonstrates that an enhanced concentration of analyte at the surface is not due to merely the establishment of an electrical double layer at the glycerol surface but results from the formation of intimate ion pairs. Support for this hypothesis is provided by the observation of a peak that represents the $[M - 2H + ON]^-$ ion at m/z 933 for the dodecasodium salt of inositol hexaphosphate (Figure 7b).

The analytes discussed in this article most often are analyzed by NI-FAB-MS, although they can form positive ions as well. If ONBr simply yields a higher concentration of analyte at the surface of the FAB matrix, then the presence of ONBr should also result in enhancement of the analyte's positive ion signal by the same amount. Of all the compounds tested, only acetyl CoA showed an increase in the PI-FAB-MS response with the addition of ONBr. For other analytes, an increase in the response of ONBr-assisted NI-FAB was not accompanied by a similar result in the positive ion mode. One possible explanation for this observation is that the chemical environment created in the presence of ON^+ assists in desorption of the analyte as singly charged anions but not as singly charged cations. Alternatively, it may suggest that the simple idea of an analyte concentration gradient created by ON^+ may be insufficient to explain the results.

Other mechanisms are certainly possible. When ONBr, glycerol, and analyte are mixed together, the formation of a layer of surfactant on the surface may not be instantaneous but diffusion limited. It has been pointed out that diffusion in glycerol is very slow [51] —too slow for such effects to be seen on the time scale of the experiment. If this is the case, then ONBr may not enhance the desorption of the analyte by providing an enrichment at the surface, but instead may promote ionization in some way. That is, ion pairs may be formed from ON^+ and analyte ions, and $[M - H]^-$ ions may be generated more readily from such complexes, but they would be distributed throughout the solution and not concentrated at the surface. Although this scheme is attractive because it does not require rapid diffusion through a viscous matrix, it is not consistent with the temporal observations discussed above.

No one mechanism completely accounts for all of the phenomena observed in this study. For example, ONBr does not improve the NI-FAB response for all types of polyanionic species. Analytes with one or more phosphate groups show the largest enhancement, whereas some classes of compounds that contain mul-

tiple carboxylic acid groups, such as polysialogangliosides and sialic acid oligomers, exhibit little or no increase in signal with the addition of ONBr. In the absence of surfactant, gangliosides may already have a large surface concentration as a consequence of their lipophilic structure; however, they do not produce strong signals in FAB. Surfactant molecules could actually compete with gangliosides for sites at the surface of the matrix and suppress the analyte signal. Conversely, sialic acid oligomers are not expected to be surface active because they are quite hydrophilic; consequently, the presence of a surfactant should improve their detectability. This suggests that specific structural features may interfere with the formation of analyte–surfactant complexes. These observations are inconsistent with enhanced desorption in which a surface layer of ON^+ leads to the concentration of analyte molecules at the surface. According to this simple description, the same enhancement should be observed for all polyanions regardless of their composition. Instead, it implies that the addition of ONBr creates a specific chemical environment that is highly favorable for the desorption of polyphosphates as singly charged anions, but much less effective for other types of polyanions.

Signal duration also varied widely from sample to sample. Some compounds produced extremely high peak intensities in NI-FAB in the presence of ONBr, but the signals persisted for shorter periods of time than in conventional FAB experiments. Whereas this may be attributed to a transient accumulation of analyte at the surface, it was not found to be true for all compounds. Some analytes such as phosphopeptides showed a similar enhancement, yet the signal levels were quite stable and lasted more than 15 min. None of the proposed mechanisms can adequately explain such disparity in the temporal dependence of the signal for different analytes.

The possibility of the formation of concentration gradients in the FAB matrix was investigated further in a sample preparation study. Considering that diffusion in glycerol is slow, the analysis of a homogeneous sample should yield a lower signal level than a sample prepared with a surface layer of ONBr. A number of techniques for construction of the FAB sample were tested. The order of application of solutions to the probe tip was varied, some of which resulted in a homogeneous mixture and others with a layer of ONBr deposited on the surface of the glycerol. No significant differences in the signal levels were observed, which indicates that the mechanism responsible for enhancement is not dependent on a pre-applied surface layer of surfactant.

The quantity of ONBr present in the sample was found to influence the NI-FAB response. In preliminary studies with acetyl coenzyme A, it was noted that an increase in the amount of ONBr added to the sample was followed by a concomitant increase in the absolute intensity of the $[M - H]^-$ ion. As mentioned

previously, the maximum response occurred for an ONBr–acetyl coenzyme A mole ratio of about 4:1 (Figure 9). This suggests the possibility of ion-pair formation between the ON^+ ion and each of the four anionic sites generated by dissociation of the acidic hydrogens in the molecule. However, data from an analogous study with EDTA do not lead to the same conclusion. The pseudomolecular ion for two different amounts of EDTA was monitored as a function of the stoichiometric amount of ONBr. In both cases, the maximum intensity of the peak that represents the $[M - H]^-$ ion was generated with the addition of 5–6 nmol ONBr to the sample, which resulted in different values for the optimum mole ratio. If signal enhancement were governed by simple charge interaction, then the optimum ONBr–analyte mole ratio would be proportional to the number of anionic sites in a given molecule. These data would be most consistent with an enhanced-ionization mechanism, which indicates that a threshold exists for the minimum amount of ONBr required for signal enhancement. The slight decrease in response observed at mole ratios higher than the optimum could be due to the formation of surfactant micelles, which changes the location of analyte–surfactant complexes from the surface of the liquid to the interior of the droplet.

Other experimental observations provide insight into the nature of the FAB target in these experiments. When the N-alkylnicotinium halides were prepared with shorter alkyl chains (C_1–C_5), the surfactant effect was significantly reduced. Enhancement of the NI-FAB response was much lower with these additives than with that produced by ONBr. At the extreme (i.e., $R = C_1$–C_3), no increase in signal was observed. Relative surface activity should increase as the alkyl chain length increases [26], so the enhancement is likely dependent on this physical property in some way. Even for $R = CH_3$, ion pairs should be formed and higher signal intensities should be detected if the enhanced-ionization mechanism is operative. Thus, this particular observation that involves alkyl chain length is consistent with the surface activity mechanism.

The strongest criticism of the surface activity model is probably the slow rates of diffusion in glycerol. Although textbooks present a simple description of the FAB target as two components—analyte in the viscous liquid matrix—the actual composition of the sample is quite different. Usually solid samples are not introduced directly into glycerol for analysis of the resultant solution by FAB. To facilitate deposition of precise amounts of matrix, analyte, and surfactant (in our experiments) onto the FAB probe tip, all are delivered via a solvent. Thus the initial solution consists of methanol, water, glycerol, analyte, and surfactant. Methanol, water, and glycerol constitute the bulk of the solution; analyte and surfactant are minor components. We assume that the volatile solvents are lost quickly both during sample preparation and upon introduction into the vacuum system; however, the

initial solution on the FAB probe tip must be considered. The approximate viscosity of a freshly prepared sample was calculated by extending Snyder's formula for an ideal binary mixture [52] to a ternary mixture. By using the volume fractions for a typical analysis and neglecting contributions from the analyte and ONBr, the viscosity of the solution was estimated to be 1.43 cP. When compared to pure glycerol (1490 cP) and pure water (1.002 cP), diffusion coefficients in this mixture are expected to be similar to those measured in aqueous systems. Thus the diffusion rates in glycerol are not relevant and it is reasonable to expect rapid diffusion of ON^+ to the surface of the matrix.

The mechanism by which volatile solvents are lost may also contribute to the formation of a surface layer of ON^+ and analyte complexes. If the initial solution of the FAB probe tip is considered to be a collection of small volume elements, then a volume element that is internal to the droplet is relatively unaffected by the low pressure encountered upon introduction into the mass spectrometer. However, a volume element on the surface of the droplet would lose volatile solvent molecules most quickly. As this process of solvent release continues to occur at the surface, the concentrations of analyte and additives increase as the total volume decreases. The dynamics of this method are extremely complex because azeotropes can be formed, the composition of the solution that undergoes fast-atom bombardment is not known exactly, and conditions change throughout the course of an experiment. However, when the details of sample preparation are considered and the solution and evaporation dynamics are taken into account, one could certainly conclude that a simple mechanism that involves true surface activity is operative in these experiments. If this is the case, ONBr analogs that involve very different basic sites other than nitrogen may be the key to enhancement of the NI-FAB-MS response for other types of analytes that form polyanionic species in solution.

Acknowledgments

References

1. Schram, K. H. In *Biomedical Applications of Mass Spectrometry*; Suelter, C. H.; Watson, J. T., Eds. Wiley Interscience: New York, 1990; pp 203–287.
2. Newton, R. P. *Rapid Commun. Mass Spectrom.* 1993, 7, 528–537.
3. Moser, H.; Wood, G. W. *Biomed. Environ. Mass Spectrom.* 1988, 15, 547–551.
4. Sandstrom, A.; Chattopadhyaya, J. *J. Chem. Soc., Chem. Commun.* 1987, 862–864.
5. Viari, A.; Ballini, J. P.; Vigny, P.; Shire, D.; Dousset, P. *Biomed. Environ. Mass Spectrom.* 1987, 14, 83–90.
6. Hogg, A. M.; Kelland, J. G.; Vederas, J. C.; Tamm, C. *Helv. Chim. Acta* 1986, 69, 908–917.
7. Eagles, J.; Javanaud, C.; Self, R. *Biomed. Mass Spectrom.* 1984, 11, 41–46.
8. Grotjahn, L.; Blocker, H.; Frank, R. *Biomed. Mass Spectrom.* 1985, 12, 514–524.
9. Grotjahn, L.; Frank, R.; Blocker, H. *Int. J. Mass Spectrom. Ion Phys.* 1983, 46, 439–442.
10. Grotjahn, L.; Frank, R.; Blocker, H. *Nucleic Acid Res.* 1982, 10, 4671–4678.
11. Ii, T.; Okuda, S.; Hirano, T.; Tsujimoto, K.; Ohashi, M. *Org. Mass Spectrom.* 1993, 28, 127–131.
12. Clifford, A. J.; Silverman-Jones, C. S.; Creek, K. E.; DeLuca, L. M.; Tondeur, Y. *Biomed. Mass Spectrom.* 1985, 12, 221–227.
13. Fenselau, C.; Feng, P. C. C.; Chen, T.; Johnson, L. P. *Drug Metab. Dispos.* 1985, 10, 316–318.
14. Sauer, F. D.; Blackwell, B. A.; Kramer, J. K. G.; Marsden, B. J. *Biochemistry* 1990, 29, 75–93.
15. Sherman, W. R.; Ackermann, K. E.; Berger, R. A.; Gish, B. G.; Zinbo, M. *Biomed. Environ. Mass Spectrom.* 1986, 13, 331–341.
16. Linhart, R. J.; Wang, H. M.; Loganathan, D.; Lamb, D. J.; Mallis, L. M. *Carbohydr. Res.* 1992, 225, 137–145.
17. Tip, L.; Heerma, W.; Contreras, R. R.; Kamerling, J. P. *Biol. Mass Spectrom.* 1991, 20, 94–97.
18. Heerma, W.; Versluis, C.; Kulik, W.; Contreras, R. R.; Kamerling, J. P. *Biomed. Environ. Mass Spectrom.* 1988, 17, 257–263.
19. Rouse, J. C.; Allison, J. *J. Am. Soc. Mass Spectrom.* 1993, 4, 259–269.
20. De Pauw, E.; Agnello, A.; Derwa, F. *Mass Spectrom. Rev.* 1991, 10, 283–301.
21. DePauw, E. *Mass Spectrom. Rev.* 1986, 5, 191–212.
22. Barber, M.; Bordoli, R. S.; Elliot, G. J.; Sedgwick, R. D.; Tyler, A. N. *J. Chem. Soc., Faraday Trans. 1* 1983, 79, 1249–1255.
23. Barber, M.; Bordoli, R. S.; Elliot, G. J.; Sedgwick, R. D.; Tyler, A. N. *Anal. Chem.* 1982, 54, 645A–657A.
24. Busch, K. L.; Unger, S. E.; Vincze, A.; Cooks, R. G.; Keough, T. *J. Am. Chem. Soc.* 1982, 104, 1507–1511.
25. Clench, M. R.; Garner, G. V.; Gordon, D. B.; Barber, M. *Biomed. Mass Spectrom.* 1985, 12, 355–357.
26. Ligon, W. V. In *Biological Mass Spectrometry*; Burlingame, A. L.; McCloskey, J. A., Eds. Elsevier Science Publishers: Amsterdam, 1989; pp 61–75.
27. Ligon, W. V.; Dorn, S. B. *Int. J. Mass Spectrom. Ion Processes* 1986, 78, 99–113.
28. Ligon, W. V.; Dorn, S. B. *Int. J. Mass Spectrom. Ion Processes* 1984, 57, 75–90.
29. Ligon, W. V.; Dorn, S. B. *Int. J. Mass Spectrom. Ion Processes* 1984, 61, 113–122.
30. Falick, A. M.; Maltby, D. A. *Anal. Chem.* 1989, 182, 165–169.
31. Stoll, M. S.; Mizuochi, T.; Childs, R. A.; Feizi, T. *Biochem. J.* 1988, 256, 661–664.
32. Ligon, W. V.; Dorn, S. B. *Fresenius Z. Anal. Chem.* 1986, 325, 626–627.
33. Pavlovich, J. G.; Yen, T.-Y.; Barofsky, D. F.; Kopniczky, J.; Sundqvist, B. U. R *Proceedings of the 40th ASMS Conference on Mass Spectrometry and Allied Topics*; Washington, DC, May 31–June 5, 1992; pp 543–544.
34. Musser, S. M.; Kelley, J. A. *Proceedings of the 39th ASMS Conference on Mass Spectrometry and Allied Topics*; Nashville, TN, May 19–24, 1991; pp 374–375.
35. Grubb, M. F.; Musser, S. M. *Proceedings of the 39th ASMS Conference on Mass Spectrometry and Allied Topics*; Nashville, TN, May 19–24, 1991; pp 376–377.
36. Shaefer, J. A.; Jarboe, C. H. *J. Med. Chem.* 1970, 13, 1026–1027.
37. Wheelan, P.; Zirrolli, J. A.; Clay, K. L. *Proceedings of the 39th ASMS Conference on Mass Spectrometry and Allied Topics*; Nashville, TN, May 19–24, 1991; pp 1023–1024.

38. Millington, D. S.; Maltby, D. A.; Farrow, P. In *Advances in Mass Spectrometry*, Vol. 10B; Todd, J. F. J., Ed. Wiley & Sons: Chichester, U.K., 1985; pp 591–592.

39. Millington, D. S.; Maltby, D. A. *Proceedings of the 32nd ASMS Conference on Mass Spectrometry and Allied Topics*; San Antonio, TX, May 27–June 1, 1984; pp 398–399.

40. Deprun, D.; Szabo, L. *Rapid Commun. Mass Spectrom.* 1989, *3,* 171–175.

41. Orlando, R. *Anal. Chem.* 1992, *64,* 332–334.

42. Shimanskas, C.; Ng, K.; Karliner, J. *Rapid Commun. Mass Spectrom.* 1989, *3,* 300–301.

43. Moon, D.-C.; Kelley, J. A. *Biomed. Environ. Mass Spectrom.* 1988, *17,* 229–237.

44. Li, Q. M.; Dillen, L.; Claeys, M. *Biol. Mass Spectrom.* 1992, *21,* 408–410.

45. Beavis, R. C.; Chait, B. T. *Anal. Chem.* 1990, *62,* 1836–1840.

46. Crow, F. W.; Tomer, K. B.; Gross, M. L.; McCloskey, J. A.; Bergstrom, D. E. *Anal. Biochem.* 1984, *139,* 243–262.

47. Pourfarzam, M.; Bartlett, K. *J. Chromatogr.* 1991, *570,* 253–276.

48. Walton, T. J.; Hughes, S. Y. *Rapid Commun. Mass Spectrom.* 1993, *7,* 555–560.

49. Naohara, J.; Manabe, M. *J. Chromatogr.* 1992, *603,* 139–143.

50. Davis, K. R.; Darvill, A. G.; Albersheim, P.; Dell, A. *Plant Physiol.* 1986, *80,* 568–577.

51. Kriger, M. S.; Cook, K. D.; Short, R. T.; Todd, P. J. *Anal. Chem.* 1992, *64,* 3052–3058.

52. Snyder, L. R.; Kirkland, J. J. *Introduction to Modern Liquid Chromatography*, 2nd ed. John Wiley: New York, 1979; pp 836–838.