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Studies of Naturally Occurring Modifications of Sialic Acids by Fast-atom Bombardment-Mass Spectrometry

ANALYSIS OF POSITIONAL ISOMERS BY PERIODATE CLEAVAGE*

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A variety of modifications of sialic acids have been described in nature. There are currently many difficulties in the detection and quantitation of these modified sialic acids from biological sources. We report here that fast-atom bombardment-mass-spectrometry (FAB-MS) of native sialic acids provides specific detection and quantitation of many previously known compounds. Derivatization of the sialic acids by reduction and peracylation under acidic conditions prior to FAB-MS provides further confirmation of their identity and improves the sensitivity of detection. Samples containing as little as 100 ng of a derivatized sialic acid loaded onto the FAB target allowed accurate identification. Mixtures of sialic acids could be analyzed, and minor components were seen, at levels undetectable by other currently known techniques. Analysis of known mixtures of different sialic acids gave reproducible relative signal intensities, indicating that quantitative data can be derived from the FAB-MS spectra.

After reduction and peracylation, each sialic acid gave two major molecular ions, corresponding to the fully derivatized linear species and a lactone form, and a minor ion, corresponding to an anhydro form. Lactone formation was minimal in the case of four substituted sialic acids, indicating that the hydroxyl group at the 4-position is involved in lactonization. Differentiation between different positional isomers of the modified sialic acids could be achieved using controlled degradation with periodate, tagging of the fragments with *p*-aminobenzoic acid ethyl ester under acid reducing conditions, peracylation, and FAB-MS of the derivatized products. We used this FAB-MS strategy to identify a novel sialic acid, 8-*O*-methyl-7,9-di-*O*-acetyl-*N*-glycolyl-neuraminic acid from the starfish *Pisaster brevispinus*, and to demonstrate the presence of a previously undetected sialic acid, 4,8-anhydro-*N*-acetylneuraminic acid in acid hydrolysates of horse serum. We also use FAB-MS to show that the alkaline conditions traditionally used for analytical de-*O*-acetylation of sialic acids causes substantial conversion of 4-*O*-acetylated sialic acids into the same anhydro compound.

The sialic acids are a family of 9-carbon carboxylic acids which have tissue-specific and developmentally regulated distribution (1-6). More than 25 different kinds of modified sialic acids have now been reported in nature. Many of these sialic acids arise from substitution of the parent molecule with a variety of different groups (see Fig. 1, reviewed in Refs. 7 and 8). These modifications have been shown to affect a wide spectrum of biological phenomena (7-16). This is not surprising, since many of the substituents are relatively large compared with the parent molecule itself (see Fig. 1). However, because of prior technical limitations, many studies of the sialic acids have failed to take into account this structural diversity. In order to explore the biology of these modifications it is necessary to release, purify, identify, and quantify the various sialic acids in complex biological mixtures.

It is now possible to release and purify many of these compounds from biological sources in an intact state (17, 18). However, further study of the purified compounds is limited by difficulties in identification and precise quantification, particularly when the molecules are present in small quantities and in mixtures. NMR spectroscopy can provide precise identification of many sialic acids. However, this analysis requires much material, and the analysis of mixtures of more than two components becomes very difficult (8, 17, 19). Gas-liquid chromatography/mass spectrometry (GLC/MS)[1] was originally used with great success to identify many of the modified sialic acids (7, 17, 20, 21). However, this type of analysis is complicated by highly variable degrees of derivatization, variable recovery from the GLC column and different detector responses of the various sialic acids. Characterization by high pressure liquid chromatography (HPLC) suffers from the overlapping elution positions of several of the compounds and the lack of specificity in detection of sialic acids in most

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[1] The abbreviations used are: FAB-MS, fast atom bombardment-mass spectrometry; HPLC, high pressure liquid chromatography; GLC/MS, gas-liquid chromatography/mass spectrometry; TLC, thin layer chromatography; BSM, bovine submaxillary mucin; ABEE, *p*-aminobenzoic acid ethyl ester; TBA, 2-thiobarbituric acid. The various sialic acids are designated according to Schauer (7, 17) and others using combinations of Neu (neuraminic), Ac (acetyl), Gc (glycolyl), and Me (methyl); *e.g.* Neu5,7Ac_2 is 7-*O*-acetyl-*N*-acetylneuraminic acid.

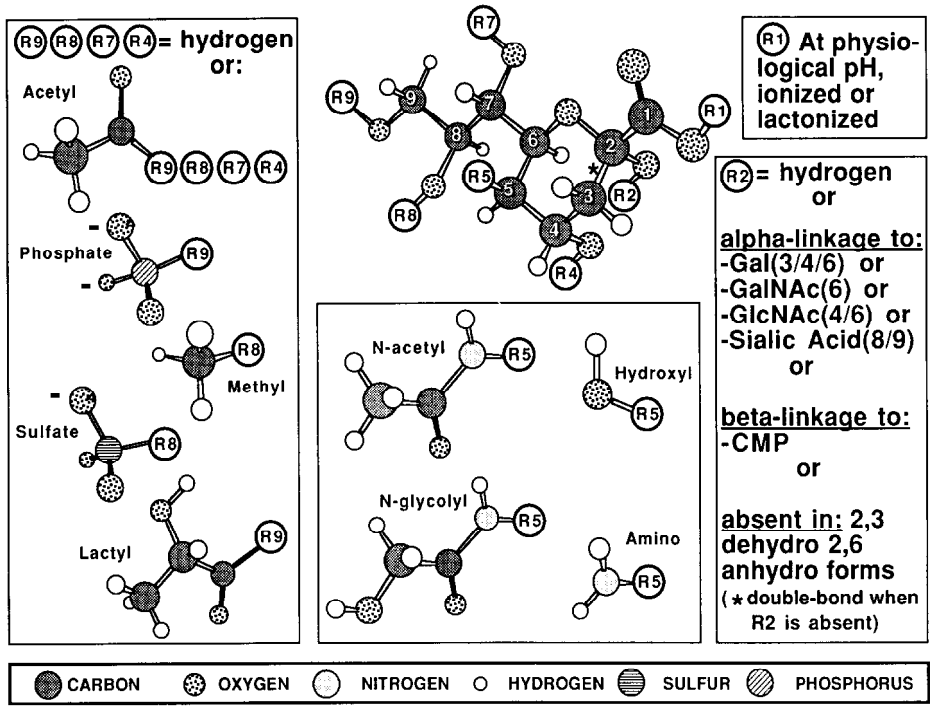

FIG. 1. **The sialic acids.** The 9-carbon backbone of the sialic acids is shown in chair conformation. The various substitutions described to date in nature (at R_4, R_5, R_7, R_8, and R_9) are indicated. Additional diversity is generated by various types of glycosidic linkage (at R_2), by the generation of lactones (at R_1), and by dehydro forms (eliminating R_2).

methods (absorbance at 200–210 nm) (22–26). Direct inlet HPLC-mass spectrometry has been demonstrated for underivatized *N*-acetyl-neuraminic acid (Neu5Ac) (17). However, this approach is less sensitive than capillary GLC/MS and has not been explored further.

In order to unambiguously identify and quantify sialic acid modifications in mixtures obtained from biological sources in very small quantities, we explored the use of fast atom bombardment-mass spectrometry (FAB-MS) (27). There is a single previous report of putative tri-*O*-acetylated Neu5Ac (28) identified by FAB-MS without derivatization. We report here for the first time the use of this technique for the detailed analysis of a variety of sialic acids in the native and derivatized states and its application for the detection of some new compounds.

EXPERIMENTAL PROCEDURES[2]

RESULTS AND DISCUSSION

Sources and Purification of Sialic Acids—Naturally occurring sialic acids were isolated from bovine submaxillary gland, equine serum, and starfish gangliosides by mild acid hydrolysis and submitted to several purification steps (see "Experimental Procedures"). The conditions for acid hydrolysis were adjusted for each tissue source to optimize release of intact modified sialic acids. For release of side-chain (7/8/9) *O*-acetylated sialic acids, hydrolysis in 2 M acetic acid at 80 °C gave adequate release with minimal loss and migration of the *O*-acetyl groups (18). Since others have found that these conditions give poor yields of 4-*O*-acetyl sialic acids (32), such molecules were released with formic acid (pH 2.0) at 70 °C (17). In each case, repeated hydrolysis following removal of the released sialic acids gave maximum yields, with minimum destruction of *O*-acetyl esters. All purification procedures were performed at 4 °C promptly after the release, and the purified products were stored dry in aliquots at −20 °C prior to analysis. In the purification, the weak ion exchanger Dowex 3x4A was used in preference to the more conventional Dowex-1 resin, and preparative HPLC (at room temperature) was used in place of gradient elution from a Dowex-1 column (17, 21). These changes were particularly important to minimize the loss and migration of *O*-acetyl groups (18). Shown in Fig. 2A (Miniprint) is the elution profile from a cellulose column of the mixture of sialic acids obtained from bovine submaxillary mucin (BSM), monitored by the 2-thiobarbituric acid (TBA) reaction for sialic acids. The presence of side-chain (7/8/9) *O*-acetyl esters was monitored by carrying out the TBA reaction before and after base treatment (these sialic acids give a decreased response prior to de-*O*-acetylation). Individual fractions were also monitored by HPLC to determine the types of sialic acids present. Shown in Fig. 2*B* is a composite result of all the HPLC runs of individual fractions. The composite profile is shown as the percent of each sialic acid species present in each fraction, calculated from the integration of different peaks in individual HPLC runs. Based upon these results, fractions were pooled as needed, and subjected to preparative HPLC fractionation. Some examples of the purification achieved by one HPLC step after cellulose chromatography are shown in Fig. 3 (for separation of Neu5,7-(8),9Ac$_3$ from Neu5,9Ac$_2$, and separation of Neu5,7(8),9Ac$_3$ from Neu5,7,8,9Ac$_4$). In some cases, repeated HPLC fractionation was necessary to achieve purity (approximately >90%, data not shown). Fig. 4*A* shows the profile of the cellulose chromatography of the sialic acids of equine serum monitored by the TBA reaction, before and after de-*O*-acetylation. All fractions were monitored by TLC before and after de-*O*-acetylation by ammonia vapors. Every fourth fraction was directly analyzed by FAB-MS following derivatization as described below. Fig. 4, *B* and *C* show examples of the FAB-MS spectra of selected fractions. These are discussed in detail in the following sections. The unexpected finding of a *decreased* response in the TBA reaction after saponification is also addressed below.

Detection of Mass Ions of Native Sialic Acids by FAB-MS in Negative and Positive Ion Modes—Pure sialic acids isolated

[2] Portions of this paper (including "Experimental Procedures," Figs. 2, 3, 6, 8, 11, 13, 14, and Table 3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

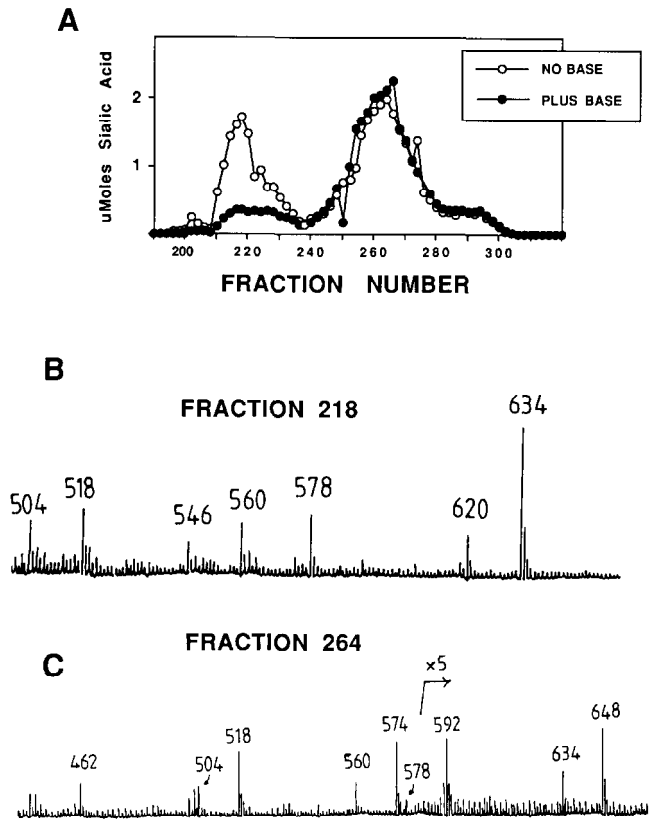

FIG. 4. **Cellulose chromatography of equine serum sialic acids, monitored by FAB-MS.** Sialic acids from ES were released, purified, and fractionated on a cellulose column as described under "Experimental Procedures." Every other fraction was monitored by the TBA assay before and after de-*O*-acetylation (*panel A*). Every fourth fraction was analyzed by positive FAB-MS after reduction and perpropionylation as described under "Experimental Procedures." Examples of spectra obtained from fractions 218 and 264 are shown in *panels B* and *C*, respectively. Fraction 218 consists mostly of Neu4,5Ac$_2$, and fraction 264 mainly contains Neu5Ac. The relevant ions are indicated in Table II.

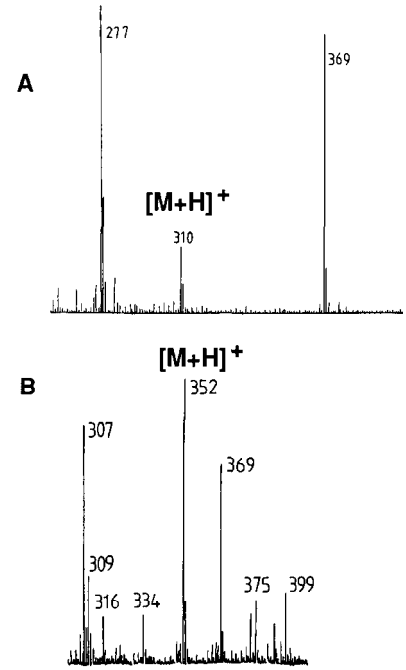

FIG. 5. **Positive FAB mass spectrum of native sialic acids.** Free sialic acids were purified and subjected to FAB-MS analysis as described under "Experimental Procedures." The spectra shown are for Neu5Ac (*panel A*) and Neu5,9Ac$_2$ (*panel B*).

and purified from bovine submaxillary mucin and equine serum were dissolved in 5% acetic acid, directly loaded into the FAB target, and submitted to both positive and negative atom bombardment as described under "Experimental Procedures." In general the chemical noise in the negative ion mode is somewhat lower than in the positive mode, and it is easier to see minor components. Fig. 5 shows examples of the spectra obtained in the positive mode for Neu5Ac and Neu5,9Ac$_2$. Each gave the expected molecular ion (*m/z* 310 and 352, respectively, in addition to matrix signals, *e.g. m/z* 277 and 369). A summary of the data obtained for several different native sialic acids in both ion modes is shown in Table I. In each case, the predicted molecular ions were unambiguously detected. Such FAB spectra of native sialic acids could be easily obtained with low microgram amounts of purified samples. For example, the spectrum in Fig. 5*A*, was obtained with 1 μg of *N*-acetylneuraminic acid (Neu5Ac). It can be seen that with this low amount of sample the matrix signals become prominent. However, the signal-to-noise ratio is still quite good.

We next studied mixtures of native sialic acids (1–5 μg total) from the same sources, containing different number of sialic acids and relative amounts of each component. These experiments showed that it was possible, even working in the microgram range to recognize the presence of several products in a given sample. Fig. 6 shows such an example. This FAB-mass spectrum of a mixture of sialic acids isolated and purified from BSM shows the presence of at least three different components. Again, there are several unidentified peaks in this low mass range.

Analysis of Sialic Acids by FAB-MS after Derivatization—In order to improve sensitivity, the FAB-MS spectra of the pure sialic acids and of different mixtures was also studied after various derivatizations. Derivatization improves sensitivity in several ways. First, it allows the extraction of interfering water-soluble contaminants. Second, it shifts the mass up to a region where the chemical noise is smaller, permitting the confident detection of small signals from the sample. Finally, it is likely that sensitivity is helped by reduction of H-bonding. Direct peracylation of native sialic acids did not produce the best sensitivity because of the presence of a series of related molecular ions and because the molecule proved to be difficult to peracylate cleanly and completely. Reduction under acid conditions followed by peracylation resulted in the best spectra. Acetic, deuteroacetic, or propionic acids can be used as acylating reagents, by forming mixed anhydrides with trifluoroacetic anhydride (36). When propionic acid is used, all significant sample-derived signals from intact sialic acids remain at even mass irrespective of the number of natural acetates. This helps sensitivity because most impurity signals are at odd masses, and the chemical noise at low mass shows more intense signals for the odd masses. Also the propionyl derivative is at higher mass than the deuteroacetyl, improving signal-to-noise ratio. In the resulting spectra, a very diagnostic pair of signals were obtained, corresponding to the molecular ions of the reduced acylated molecule and the corresponding lactone (see Fig. 7). The molecular ions (in the positive and negative ion modes) for the different derivatives of several naturally occurring sialic acids are listed in Table I. Lactonization appears to occur primarily between the hydroxyl group on C-4 and the carboxyl group, as proved by the fact that the lactone peak (*m/z* 560) is markedly reduced in the spectrum of Neu4,5Ac$_2$, which has an acetyl group at position 4 (Fig. 4*B*).

The sensitivity of detection after derivatization is also

TABLE I

Molecular ions, in the positive and negative ion modes, of native sialic acids and their reduced and perdeuteroacetylated or perpropionylated derivatives

Sialic acid	Native		Perdeuteroacetylated	Perpropionylated
	$(M + H^+)$	$(M - H^+)$	$(M + H^+)$	$(M - H^+)$
Neu5Ac	310	308	582 (O)[a]	648 (O)
			519 (L)[b]	574 (L)
Neu5Gc	326	324	643 (O)	720 (O)
			580 (L)	646 (L)
Neu5,9Ac_2 or Neu5,7Ac_2	352	350	579 (O)	634 (O)
			516 (L)	560 (L)
Neu4,5Ac_2	352	350	579 (O)	634 (O)
Neu5,7(8),9Ac_3	394	392	576 (O)	620 (O)
			513 (L)	546 (L)
Neu5,7,8,9Ac_4	436	434	573 (L)	606 (O)
			510 (L)	532 (L)
Neu4Ac5Gc	368	366	640 (L)	706 (O)
Neu5Gc9Ac	368	366	640 (O)	706 (O)
			577 (L)	632 (L)
Neu5Gc7(8),9Ac_2	410	408	637 (O)	692 (O)
			574 (L)	618 (L)
Neu5Gc7,8,9Ac_4	452	450	634 (O)	678 (O)
			571 (L)	618 (L)
Neu5Gc8Me	340	338	612 (O)	678 (O)
				604 (L)
Neu5Gc8Me9Ac	382	380	609 (O)	664 (O)
				590 (L)
Neu5Gc8Me7,9Ac_2	424	422	603 (O)	650 (O)
				576 (L)

[a] (O), open chain.
[b] (L), lactone.

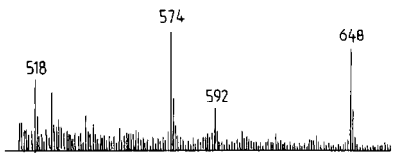

FIG. 7. **Sensitivity of detection of derivatized sialic acids by FAB-MS.** The positive FAB-mass spectrum of 100 ng of Neu5Ac is shown after reduction and perpropionylation. The major signals seen are *m/z* 648 (linear form) and *m/z* 574 (lactone form).

demonstrated by the spectrum in Fig. 7. This spectrum was obtained with only 100 ng of sample loaded on the FAB target, after reduction and derivatization of 0.5 μg of pure Neu5Ac. Unlike the case with the native molecule, the major signals seen are those arising from the compound under study (compare with Fig. 5*A*, where 10 times the amount of native Neu5Ac was used). The major signals seen are *m/z* 648 (open chain form) and *m/z* 574 (lactone form). The signal at *m/z* 518 likely results from an anhydro form, which is discussed later. As demonstrated in Fig. 8, the increased sensitivity achieved by derivatization is particularly useful in the analysis of a complex mixture of sialic acids. The spectrum in Fig. 8*A* was obtained from a native sialic acid fraction isolated from BSM after cellulose column chromatography and preparative HPLC. The reduced perpropionylated derivatives of the same mixture are shown in Fig. 8*B*. The native spectrum shows a major $[M\text{-}H^+]^-$ at 352 corresponding to Neu5AcOAc. This is in keeping with the HPLC and TLC analyses, which had characterized this sample as a "pure standard" for Neu5,9Ac_2. A smaller signal at *m/z* 368 was also noted in the FAB-MS analysis, possibly corresponding to Neu5GcOAc; however, the major matrix signal at 369 precluded firm identification. On the other hand, the perpropionylated derivatives (Fig. 8*B*) were unambiguous: *m/z* 634 and 560 for Neu5AcOAc and its lactone and *m/z* 706 and 632 for Neu5GcOAc and its lactone, respectively. The higher sensitivity also allowed confident detection of another minor component, Neu5Ac (signals at 648 and 574). Thus, minor components in a pure standard that were undetectable by HPLC and TLC analyses were suspected based upon the native FAB-MS spectrum. Derivatization of the sample then allowed unambiguous detection of these minor components.

Fig. 9 illustrates further examples of the usefulness of the reduction/peracylation strategy for examining mixtures. Fig. 9*A* shows the analysis of a mixture of sialic acids obtained from BSM, which apparently contained two major components, according to HPLC analysis. The sample was analyzed after reduction and perpropionylation. The positive spectrum shows signals separated by 14 mass units corresponding to the difference between naturally occurring acetyl groups and the chemically introduced propionyl groups. The derivatives of the linear forms are at 620, 634, and 648, with the corresponding lactones at 546, 560, and 574. Therefore, this sample contains di-*O*-acetyl-Neu5Ac and mono-*O*-acetyl Neu5Ac as major components together with a previously undetected amount of non-acetylated material. Again, the unambiguous identification of the major components, and the sensitive detection of the minor components are demonstrated.

In this spectrum, the signals at *m/z* 490, 504, and 518 also show the identical mass intervals of 14 mass units. These are likely to be anhydro forms. Alternatively, they could result from failure to propionylate the C-2 hydroxyl group after lactone formation. However, the corresponding derivatives of the open chain form give very minor signals (*m/z* 564, 578, and 592), suggesting that the first explanation is most likely. The proposed structures of these three derivatives from Neu5Ac are shown as an example in Fig. 9*B*. These derivatives do not interfere with the interpretation of the spectra and can be taken into account when quantitation is important. Furthermore, the typical set of three signals separated by 74 and 56 massunits for the reduced and perpropionylated derivatives give a very characteristic pattern for a sialic acid. As

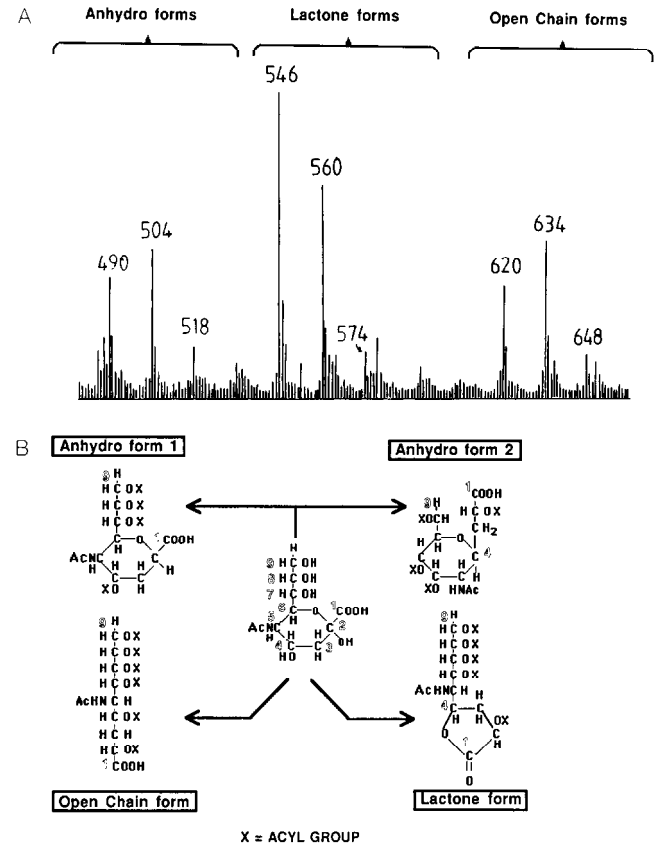

FIG. 9. **FAB spectrum of mixtures of sialic acids obtained from BSM.** *Panel A*, a sample containing two major *O*-acetylated sialic acid components was analyzed by FAB-MS after reduction and perpropionylation. According to the spectrum, this sample contains di-*O*-acetyl-Neu5Ac and mono-*O*-acetyl-Neu5Ac as major components together with a previously undetected amount of non-acetylated material. *Panel B*, proposed structures for the three characteristic ions observed during FAB-MS of reduced and peracylated Neu5Ac.

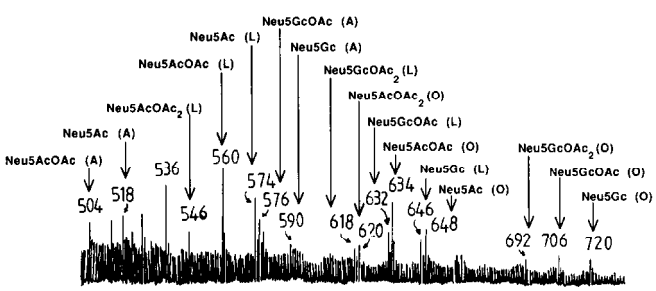

FIG. 10. **FAB spectrum of an unfractionated mixture of sialic acids, after reduction and perpropionylation.** A sample of total purified sialic acids from BSM, that was not fractionated by chromatography on cellulose, was analyzed by FAB-MS after reduction and perpropionylation. The spectrum shown contains signals for the open chain (*O*), the lactone (*L*), and the anhydro (*A*) forms from each of six different sialic acids (Neu5Ac, Neu5Gc, and their mono- and di-*O*-acetylated derivatives).

pointed out above, the 4-substituted molecules fail to give this complex pattern, since the 4-position is likely to be involved in the lactone formation.

All of the data shown above was obtained with sialic acids partially fractionated by cellulose chromatography. To examine the usefulness of reduction/perpropionylation for even more complex mixtures, we analyzed a mixture of total sialic acids from BSM without fractionation by cellulose column chromatography. The resulting spectrum is shown in Fig. 10. In contains the two major signals from each of six different sialic acids (Neu5Ac, Neu5Gc, and their mono- and di-*O*-acetylated derivatives). The anhydro form for each component is also present, although difficult to detect above the background in some cases. This spectrum also shows that purification and fractionation by cellulose chromatography is not essential prior to analysis by FAB-MS.

Quantitation of Derivatized Sialic Acids in Mixtures by FAB-MS—Mixtures containing known amounts of various modified sialic acids were prepared and submitted to several FAB experiments in order to test the reproducibility of relative signal intensities and hence the feasibility of obtaining quantitative data from the FAB experiments. For each experiment 10 nmol of total sample containing different relative amounts of Neu5Ac, Neu5Gc, and Neu5,9Ac_2 were used. Each sample was reduced and perpropionylated and positive FAB spectra obtained. Initially we averaged up to 10 consecutive scans for each sample, but we found that the averaged relative signal intensities did not differ significantly from those in the strongest spectrum acquired from each sample. Representative data are shown in Fig. 11 for three samples which were prepared by mixing standards to give anticipated Neu5Ac/Neu5,9Ac_2/Neu5Gc ratios of 1.0:1.0:1.0, 1.0:0.4:1.0, and 1.0:1.0:2.5, respectively. The FAB spectra indicate that Neu5Gc9Ac is also present (*m/z* 706 and 632) as a minor component. For quantitative purposes we summed the linear and lactone forms of each component (*m/z* 648 and 574 for Neu5Ac, *m/z* 634 and 560 for Neu5,9Ac_2, and *m/z* 720 and 646 for Neu5Gc). The data indicated that equal intensities of Neu5Ac and Neu5,9Ac_2 give comparable summed signal intensities whose relative ratio is not altered by varying the Neu5Gc content (Fig. 11, *A* and *C*); the slightly lower intensity of the Neu5,9Ac_2 signals compared with the Neu5Ac signals is likely due to the fact that the colorimetric quantitation of Neu5,9Ac_2 also detected the minor component Neu5Gc9Ac (hence there is slightly less Neu5,9Ac_2 than expected). The data also show that Neu5Gc gives signal intensities approximately 50% lower than Neu5Ac for the same amount of sample (Fig. 11, *A* and *B*), but the relative ratios are not affected by variations in the Neu5,9Ac_2 concentration. It appears that the presence of the additional substituent on the *N*-acyl side-chain reduces the surface activity of Neu5Gc derivatives relative to those of Neu5Ac, thereby giving lower abundance of ions. From several quantitative experiments such as those shown in Fig. 11, we conclude that, provided account is taken of the different molar responses of Neu5Ac and Neu5Gc, it is possible to give an estimate of the relative amounts of sialic acids present in mixtures with an error of 10–15%.

Based upon the analysis of a variety of sialic acid mixtures, in which major components were at the microgram level, we can also say that the sensitivity of FAB allows detection of very minor components (as low as 0.5% of a mixture) which would be missed by all other currently known methods.

FAB-MS of Periodate-derived Fragments of Sialic Acids—A drawback to the FAB-MS approach outlined above is that while it accurately indicates the number and type of substituents present in a molecule, it cannot differentiate between positional isomers. The special reactivity of sialic acids upon mild periodate oxidation due to the presence of one primary hydroxyl group at C-9, followed by two secondary hydroxyl groups at C-8 and C-7 is well known (37, 38). Substituents on the side-chain are known to alter this reactivity to variable extents (23, 39). By careful regulation of the experimental conditions (temperature, time, sodium metaperiodate concentration, and pH), it is possible to affect the extent of this reaction (40). Provided the adequate conditions are found, it is therefore theoretically possible to obtain a set of different

fragments from each different sialic acid isomer.

We explored different conditions for the periodate oxidation reaction followed by reductive tagging of the resulting aldehydes and elimination of the excess of reagents by extraction (data not shown). We found that optimal results were obtained when the aldehydes generated by the periodate reaction were derivatized with *p*-amino benzoic acid ethyl ester (ABEE) that can be reductively introduced at acidic pH without loss of the natural *O*-acetyl groups. The products were analyzed by FAB-MS directly after derivatization, reduction, peracylation, and extraction (see "Experimental Procedures" for details). The complete procedure can be carried out in one vial, reducing the risks of losses, and minimizing the manipulation and the time required.

When Neu5Ac was treated with 10 mM periodate at pH 5.5 for 10 min on ice, rapid removal of two carbons (C-8 and C-9) from the side-chain occurred. After tagging with ABEE, reduction and perpropionylation two major molecular ions were observed at *m/z* 549 and 493 (Fig. 12*A*). These correspond to lactonized and anhydro forms of the truncated, tagged, reduced sialic acid. The open chain truncated molecule gives a minor signal at *m/z* 623.

When more rigorous conditions of periodate treatment (3 h at 23 °C) were employed, the major product showed a loss of a 3-carbon unit (C-7, C-8, and C-9). As shown in Fig. 12*B*, the major fragment from the reduced, perpropionylated derivative gives a molecular ion at *m/z* 389 which corresponds to

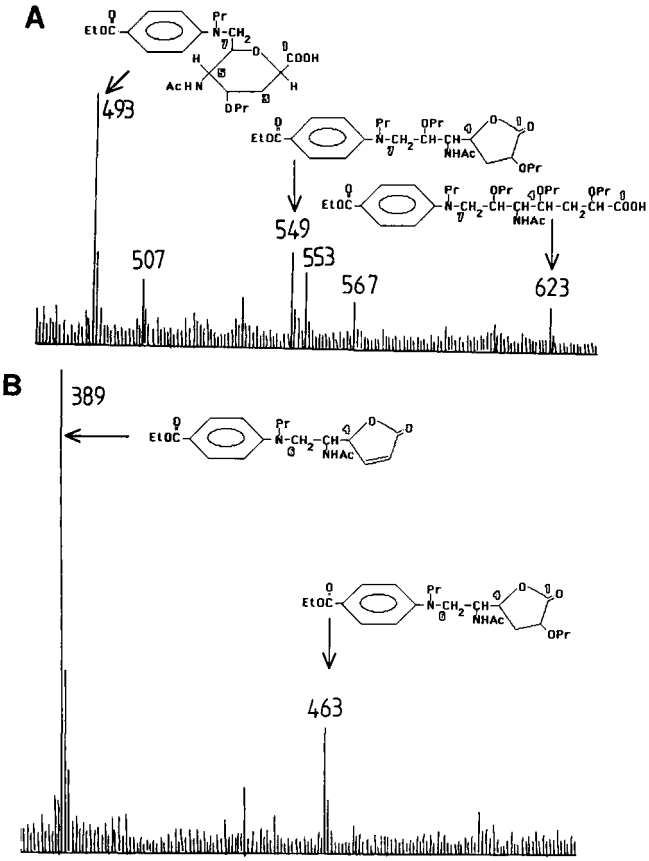

FIG. 12. **Positive FAB spectrum of Neu5Ac after periodate treatment followed by ABEE-tagging and perpropionylation.** A sample of Neu5Ac was treated with periodate and aliquots removed for ABEE-tagging and propionylation at different time points as described under "Experimental Procedures." The FAB-MS spectra are shown for the derivatives obtained after periodate treatment for 10 min on ice (*panel A*) and an additional 3 h at room temperature (*panel B*). *Pr* = *O*-propionyl group.

a dehydrated lactone. The parent lactone itself is a relatively minor component (*m/z* 463). The absence of any anhydro form after removal of C-7 supports our hypothesis that the anhydro product observed in the mild reaction is produced by nucleophilic attack of the C-6 hydroxyl at C-2. Thus, the oxidative cleavage which removes C-7 converts C-6 to the aldehyde which can no longer behave as a nucleophile to form an anhydro derivative. Predicted fragments were also seen for the peracetylated and perdeuteroacetylated derivatives of periodate-treated Neu5Ac (spectra not shown). These experiments indicated that FAB-MS could be a useful tool for monitoring periodate cleavages.

When isomers of natural mono-*O*-acetylated sialic acids were submitted to the same conditions of periodate treatment, clear differences were observed between them. Following the 10-min incubation on ice, there was essentially no cleavage of Neu5,9Ac$_2$ (therefore the product affords a mass spectrum identical to that of the starting material; *m/z* 634, 560 and 504, see Fig. 13*A*). On the other hand, rapid degradation of the other mono-*O*-acetylated sialic acids was seen, with loss of a 1-carbon fragment from Neu5,7Ac$_2$ and a two-carbon fragment from Neu4,5Ac$_2$, in each case with retention of the natural acetate. In the case of 4-*O*-acetylated Neu5Ac, two major products giving molecular ion signals at *m/z* 535 and 479, and a minor product at *m/z* 609 (see Fig. 13*B*) are observed. The last signal is assigned to a mono-acetylated, tagged, reduced, perpropionylated sialic acid which has lost a 2-carbon fragment. The two major signals have the correct masses for the corresponding lactonized and anhydro forms of the truncated Neu4,5Ac$_2$ molecule. We suggest that in this case a seven-membered lactone is produced by the reaction of the C-6 hydroxyl with C-1. The formation of this lactone would also provide a plausible explanation for lack of further cleavage of the 4-*O*-acetyl derivative under conditions which fully degrade both Neu5Ac and Neu5,9Ac$_2$ (see below). The latter compounds are able to form lactones by cyclization to C-4 (a more favorable reaction than cyclization to C-6) leaving the C-6/C-7 bond susceptible to further periodate cleavage. Because Neu4,5Ac$_2$ is unable to lactonize to C-4 it lactonizes to C-6 thereby preventing further periodate cleavage. When Neu5,7Ac$_2$ is analyzed in the same manner, the expected cleavage of C-1 with retention of the natural acetate, was observed (data not shown). However, the presence of some Neu5Gc in the same sample did not permit the firm identification of the fragment. This is because the expected molecular ions (*m/z* 621 and 695) for the cleavage of a 1-carbon unit from Neu5,7Ac$_2$ or a 2-carbon unit from Neu5Gc, are identical.

When the same mono-*O*-acetylated compounds are submitted to more rigorous conditions of oxidation (3 h at room temperature) further differences are seen. Neu5,9Ac$_2$ now shows the cleavage between C-7 and C-8, and then between C-6 and C-7, and becomes indistinguishable from the product of Neu5Ac at the same time point (Fig. 14*A*, compare with Fig. 13*A*). Unexpectedly, the Neu4,5Ac$_2$ does not show further degradation (Fig. 14*B*). As discussed above, Neu4,5Ac$_2$ is unable to lactonize to C-4 and therefore presumably lactonizes to C-6 thereby preventing further periodate cleavage. After 3 h, Neu5,7Ac$_2$ still shows only the expected loss of a 1-carbon unit with retention of the acetate. However, as pointed out above, the signals seen at *m/z* 621 and 695 could still be attributed to either a 1-carbon cleavage of Neu5,7Ac$_2$ or 2-carbon cleavage of Neu5Gc which was also present in the sample. To distinguish between these two possibilities, the sample was perdeuteromethylated (which replaces all *O*-acetyl and propionyl groups with deuteromethyl) and examined by

FAB-MS (Fig. 14*C*). A signal at *m/z* 487 corresponding to Neu5Gc that has lost a 3-carbon unit is seen. However, there is also a signal at *m/z* 548 corresponding to Neu5Ac truncated by a single carbon (arising from Neu5,7Ac_2). As expected, there is no signal at *m/z* 534 which would be due to Neu5Gc truncated by a 2-carbon unit.

Neu5,7(8),9OAc_3 remains unreacted when is treated with periodate even after 3 h at R.T. (data not shown), conditions in which Neu5Ac is completely degraded to *m/z* 389. Therefore, substitution at C-8 and C-9 results in very slow cleavage between C-6 and C-7. We predict that Neu5,7,8,9Ac_4 will be completely resistant to periodate cleavage. However, when the third *O*-acetyl group is located in C-4 (Neu4,5,7(8),9Ac_4), the loss of a 3-carbon unit (C7/8/9) can be expected after very long treatments.

Neu5Gc behaves in a similar manner to Neu5Ac on periodate degradation, *i.e.* rapid loss of a 2-carbon unit followed under more rigorous conditions by quantitative cleavage between C-6 and C-7. There is one significant difference, however, in the nature of the products observed after derivatization. In contrast to Neu5Ac, which cannot form an anhydro derivative after complete degradation, Neu5Gc affords both anhydro and lactonized products. We propose that the anhydro product is produced by nucleophilic attack of the glycolyl group at the C-5 position.

Table II summarizes the molecular ions of the fragments observed from several different sialic acids upon periodate oxidation, reduction under acidic conditions, tagging with ABEE, and peracylation with acetic, deuteroacetic and propionic acids. Table III provides proposed structures for each of these molecular ions and comments concerning their origin.

Use of the FAB-MS for Screening Partially Purified Mix-

TABLE II

Molecular ions of fragments following periodate treatment, reduction under acidic conditions, and tagging with ABEE of sialic acids as their perdeuteroacetylated, peracylated, perpropionylated, permethylated, and perdeuteromethylated derivatives

Figures in italics indicate predicted ions.

Sialic acid	Fragment		Perdeuteroacetyl	Peracetyl	Perpropionyl	Permethyl	Perdeuteromethyl
Neu5Ac	C-9	O[a]	654	639	709	527	548
		L[b]	591	579	635		
		A[c]	546	537	579		
	C-8/9	O	579	567	623	483	501
		L	516	507	549		
		A	471	465	493		
	C-7/8/9	O	504	495	537	439	454
		L	441	435	463		
		AL[d]	378	375	389		
Neu5Gc	C-9	O	715	697	781	557	581
		L	652	637	707		
		A	607	595	651		
	C-8/9	O	640	625	695	513	534
		L	577	565	621		
		A	532	523	565		
		AL	514	505	547		
	C-7/8/9	O	565	553	609	469	487
		L	502	493	535		
		A	457	451	479		
		AL	439	433	461		
Neu5,9Ac_2	C-8/9	O	579	567	623	483	501
		L	516	507	549		
		A	471	465	493		
	C-7/8/9	O	504	495	537	439	454
		L	441	435	463		
		A	378	375	389		
Neu4,5Ac_2	C-8/9	O	*576*	*567*	609	*483*	*501*
		L	*513*	*507*	535		
		A	468	465	479		
Neu4Ac5Gc	C-8/9	O	*637*	*625*	681	*513*	*534*
		L	*574*	*565*	607		
		A	*529*	*523*	533		
Neu5,7Ac_2	C-9	O	*651*	*639*	695	*527*	*548*
		L	*588*	*579*	621		
		AL	*514*	*505*	547		
Neu5Gc9Ac	C-8/9	O	*640*	*625*	695	*513*	*534*
		L	*577*	*565*	621		
		A	*532*	*523*	565		
	C-7/8/9	O	*565*	*553*	609	*469*	*487*
		L	*502*	*493*	535		
		A	*457*	*451*	479		
Neu5Gc7,9Ac_2	C-7/8/9	O	565	553	609	469	487
		L	502	493	535		
		A	457	451	479		
		AL	439	433	461		

[a] O, open chain.
[b] L, lactone.
[c] A, anhydro form.
[d] AL, anhydro lactone.

tures of Sialic Acids of Biological Origin: Detection of Unexpected and Potentially Novel Compounds—The capability of analyzing partially purified complex mixtures makes this approach suitable for primary screening of samples of biological origin. Thus, unexpected or potentially novel compounds could be observed, roughly quantitized, and even identified prior to rigorous purification and further characterization. Some examples of this are described below.

When a partially purified mixture of sialic acids isolated from the polar lipids of the starfish *Pisaster brevispinus* (5 nmol according to the TBA assay) was submitted to negative FAB mass spectrometry, three interesting molecular ions were detected, namely, 338, 380, and 422 (Fig. 15*A*). These ions correspond to the mass of a methylated form of *N*-glycolylneuraminic acid and its mono- and di-*O*-acetylated counterparts, respectively. While the first of these has been previously isolated from starfish (7, 41), and the presence of Neu5Gc8-Me9Ac previously suggested (42), the third compound (di-*O*-acetyl-mono-*O*-methyl-Neu5Gc) has never been previously described from any source.

An anhydro sialic acid was encountered unexpectedly when we monitored the cellulose column chromatography of equine serum sialic acids (see Fig. 4). Fractions eluting between the two major TBA-positive peaks were found to contain a signal of *m/z* 518, upon reduction and perpropionylation (Fig. 15*B*). This corresponds to the expected mass for an anhydro sialic acid. Further analysis (see below) suggests that this peak corresponds to 4,8-anhydro-neuraminic acid, a previously described molecule from acid hydrolysates of collocalia mucoid (43), and from chemical treatment of Neu5Ac (44).

Identification of a New Sialic Acid Using FAB-MS—Based upon the results of the preliminary screening described above, the derivatization and periodate oxidation strategies were employed to analyze the sialic acids from the polar lipids of the starfish *P. brevispinus*. The positive FAB spectrum of its reduced/perpropionylated derivative and its permethylated derivative are shown in Fig. 16. In the first case, the major peaks corresponded to the expected derivatives for the predicted compounds (*O*-methylated form of *N*-glycolylneuraminic acid (*m/z* 678 and 604) and its mono-*O*-acetylated (*m/z* 664 and 590) and di-*O*-acetylated (*m/z* 650 and 576) counterparts (Fig. 16*A*). The alkaline conditions of permethylation

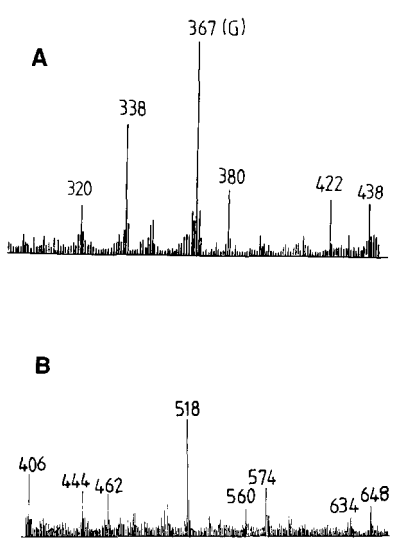

FIG. 15. **Detection of native ions of sialic acids in mixtures.** FAB-mass spectra of native sialic acids from starfish glycolipids (*panel A*, negative spectrum) or from equine serum (*panel B*, positive spectrum) are shown (see text for details).

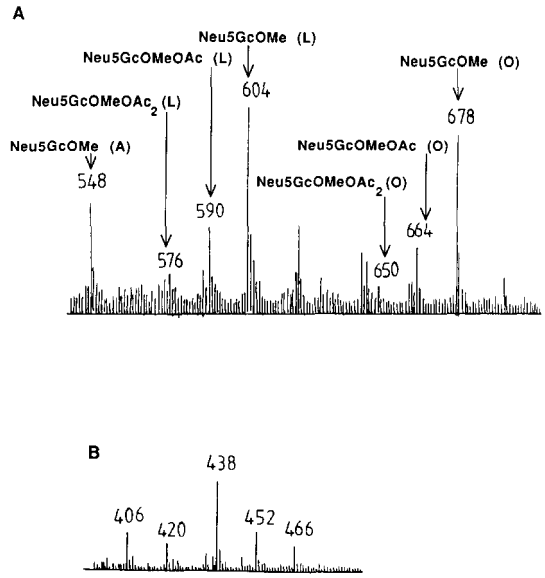

FIG. 16. **FAB spectra of sialic acids from the polar lipids of *P. brevispinus*.** The sialic acids were analyzed by FAB-MS after reduction and perpropionylation (*panel A*), and permethylation (*panel B*), as described under "Experimental Procedures."

removed the *O*-acetyl esters. The resulting spectrum (Fig. 16*B*) included *m/z* 406 (minus methanol from 438), *m/z* 420 (minus methanol from 452), and *m/z* 438 (expected mass of permethylated Neu5Gc). The signals at *m/z* 452 and 466 are overmethylated molecules that are also observed in standard Neu5Gc. This spectrum allowed differentiation between a methylated Neu5Gc type of sialic acid and a Neu5Ac molecule containing an additional CH_2OH group.

The location of the methyl group was further assessed by periodate degradation, derivatization, and FAB-MS of the mixture of sialic acids of starfish glycolipids that contained approximately 15% mono-*O*-acetylated and 5% di-*O*-acetylated components. The sample was completely resistant to mild periodate cleavage (10 min on ice, Fig. 17*A*). The signals observed are the same as those present in derivatized starting material, *i.e.* *m/z* 678, 604, and 548 for linear, lactonized, and anhydro forms of the major component (mono-*O*-methyl-Neu5Gc) together with minor signals 14 and 28 mass units lower for the mono- and di-*O*-acetylated components, respectively (compare with Fig. 16*A*). More vigorous conditions of periodate treatment (3 h at 23 °C) produced little change (see Fig. 17*B*), but there is evidence for partial loss of a 3-carbon unit giving the very minor signals at *m/z* 535, 479, and 461 corresponding to the lactonized, anhydro, and dehydrated/lactonized forms of truncated Neu5Gc. There is no evidence for removal of a 2-carbon unit prior to the 3-carbon cleavage. In comparison, similar treatment of Neu5Gc results in complete cleavage with a loss of a 3-carbon fragment (not shown). Even after more prolonged treatment with periodate (overnight at 23 °C) (Fig. 17*C*), some starting material is still present but the major signals are now derived from molecules that have lost a 3-carbon unit. Again, there is no evidence for prior loss of a 2-carbon unit (C-8/C-9). The signal at *m/z* 575 (which is also seen in the Neu5Gc spectrum, data not shown) is most likely due to a lactone component which contains one trifluoroacetyl group instead of a propionyl group (this is possible since the reagent is a mixed anhydride). This can occur if the rate *versus* stability of trifluoroacetylation does not favor replacement of a trifluoroacetic acid group by a propionyl group. Since we do not observe trifluoroacetylation in Neu5Ac, it is likely that it is the glycolyl hydroxyl group which is showing this reactivity.

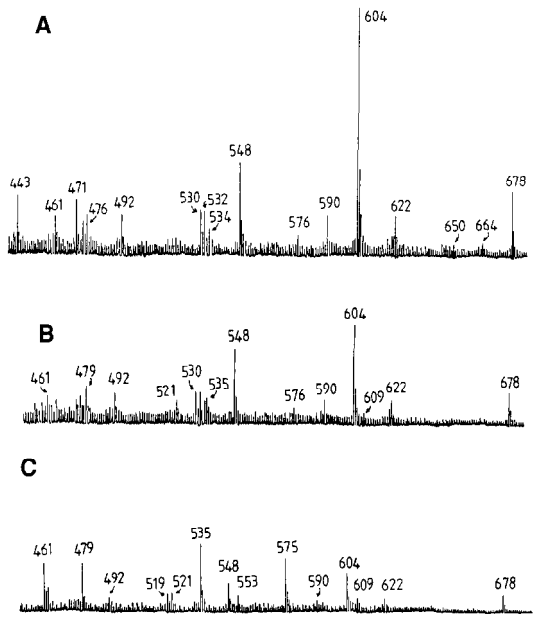

FIG. 17. **FAB spectra of starfish sialic acids after periodate oxidation.** The mixture of purified sialic acids from starfish glycolipids was treated with periodate as described under "Experimental Procedures," and aliquots were removed for ABEE tagging and propionylation after 10 min on ice (*panel A*), further 3 h at 23 °C (*panel B*), and then overnight at 23 °C (*panel C*).

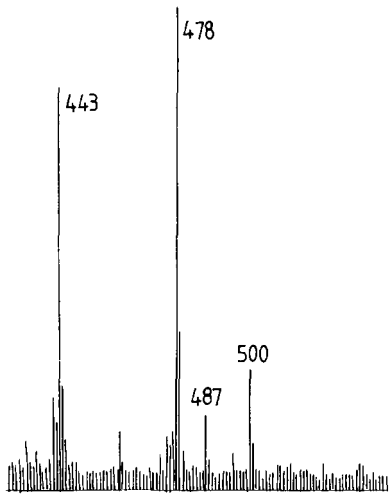

FIG. 18. **FAB spectrum of starfish sialic acids after periodate oxidation and deuteropermethylation.** The mixture of purified sialic acids from starfish glycolipids was treated for 3 h with periodate at 23 °C, ABEE-tagged, reduced, and perpropionylated, then subjected to additional deuteropermethylation, and reanalyzed by FAB-MS.

Signal assignments were corroborated by submitting the remainder of the partially cleaved and derivatized sample (after 3 h of periodate) to deuteropermethylation. This replaced each propionyl group with a CD3 group and additionally deuteromethylated the carboxyl group and the amide nitrogen. The deuterated reagent was chosen in order to differentiate introduced methyl groups from their natural counterpart. The resulting FAB spectrum (Fig. 18) contained two major signals at *m/z* 478 and 443, which we attribute to the molecular ion of the intact deuterated molecule. The minor signal at *m/z* 487 corresponds to the molecular ion for the tagged product of the periodate-cleaved material which has been truncated by a 3-carbon unit. Again, no signals corresponding to the loss of C-8 and C-9 were seen.

The resistance to periodate degradation and the fact that cleavage (when it occurs after prolonged treatment) is restricted to the C-6/C-7 bond provides convincing evidence for the location of the natural methyl group at position 8. Further, it should be noted that the mono-*O*-acetylated material is also degraded under the rigorous periodate conditions since there is no significant increase in the *m/z* 590/*m/z* 604 ratio, which would be expected if only the non-acetylated component was being degraded. Therefore the mono-*O*-acetylated compound most likely contains a 9-*O*-acetyl group. The location of the additional acetate in the di-*O*-acetylated compound cannot be directly assigned from the periodate experiment because the increase in the chemical noise in the spectra of samples treated under the very rigorous periodate conditions precludes firm conclusions regarding the abundance of a component which is only about 5% of the original sample. However, prior to periodate cleavage the ratio of lactonized to linear forms of all three components is very similar. This indicates that the additional acetate is not on position 4 (see discussion of Neu4,5Ac$_2$ above) and is presumably therefore on position 7.

Taken together, the data demonstrate the presence of a new sialic acid 8-*O*-methyl-7,9 di-*O*-acetyl-*N*-glycolylneuraminic acid and confirm the presence of 8-*O*-methyl-9-mono-*O*-acetyl-*N*-glycolylneuraminic acid in the starfish.

Further Analysis of the Anhydro Sialic Acid from Equine Serum—In the previous section, we described the unexpected finding of an anhydro sialic acid in the equine serum hydrolysate. Based upon prior literature (43, 44), we predicted that this molecule might be the previously described 4,8-anhydroneuraminic acid. To obtain further evidence for this hypothesis, the material was submitted to periodate oxidation, and FAB-MS analysis. As expected, no cleavage was observed even under drastic conditions (3 h at room temperature).

Use of FAB-MS to Monitor Analytical De-O-acetylation of Sialic Acids—We and others (18, 39) have previously described conditions for analytical de-*O*-acetylation of 9-*O*-acetylated sialic acids (0.1 N NaOH for 45 min on ice). These conditions were subsequently found to be inadequate for the de-*O*-acetylation of di- and tri-*O*-acetylated sialic acids. To obtain complete de-*O*-acetylation of these compounds, it is necessary to use 0.1 M NaOH at 37 °C for longer periods of time (26).

We therefore routinely use such conditions to eliminate interference of *O*-acetyl groups prior to analysis of free sialic acids by TBA analysis. Since *O*-acetylation of the side-chain interferes variably with the TBA reaction (7), the *increase* in reactivity following de-*O*-acetylation can also be taken as an indication that such acetylation was present. In carrying out such an analysis of acid-released sialic acids from equine serum, we were surprised to note a substantial *decrease* in reactivity in the peak that contained the 4-*O*-acetylated sialic acids (see Fig. 4*A*). We reasoned that this could be due to the formation of 4,8-anhydroneuraminic acid (43) during the base treatment. To examine this question, we subjected samples from fraction 218 (a mixture of Neu4,5Ac$_2$ and Neu4Ac5Gc) to a variety of different conditions of base treatment. We found that regardless of the conditions used, there was substantial conversion of the 4-*O*-acetylated molecule into the putative anhydro compound (see Fig. 19 for an example of a native spectrum). The signal assignments were confirmed after reduction and perpropionylation (not shown). Substantial production of the anhydro-form occurred even under conditions where only partial de-*O*-acetylation had taken place (*e.g.* 0.2 M NH_4OH, 37 °C, 30 min). It should be noted that under these conditions, the parent compounds (Neu5Ac and Neu5Gc) are relatively stable. This indicates that the formation of the anhydro compound involved the elimination

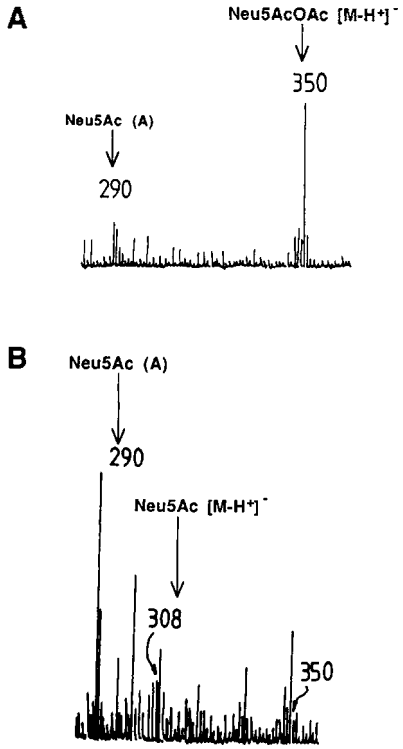

FIG. 19. **FAB spectra of native 4-*O*-acetylated sialic acids from equine serum before and after de-*O*-acetylation with alkali.** Samples of sialic acids from the equine serum enriched in Neu4,5Ac$_2$ and Neu5Gc4Ac were treated with alkali under a variety of conditions, passed over Dowex-50 (H+form), and analyzed by FAB-MS directly, and after reduction and perpropionylation. Two examples of native spectra are shown: no treatment (*panel A*), and 0.1 N NaOH at room temperature for 30 min (*panel B*).

of the 4-*O*-acetyl group. This confirms the hypothesis made by Pozsgay *et al.* (43) that 4,8-anhydroneuraminic acid can be formed from 4-substituted sialic acids. It is likely that the small quantity of 4,8-anhydroneuraminic acid seen in the acid hydrolysate also arose by a similar mechanism during acid hydrolysis.

De-*O*-acetylation of glycosidically bound sialic acids on equine serum glycoproteins followed by analysis with the TBA reaction showed a slight decrease in reactivity (data not shown). This raised the possibility that a similar reaction could take place upon bound 4-*O*-acetylated sialic acids. To explore this matter, equine serum was subjected first to de-*O*-acetylation (0.1 M NaOH at 37 °C for 30 min), dialyzed against water and then hydrolyzed with formic acid (pH 2.0) at 70 °C for 1 h or with 0.1 M hydrochloric acid at 80 °C for 1 h. The released sialic acids were purified as described under "Experimental Procedures" and analyzed by FAB-MS. The spectra showed almost complete conversion of Neu4,5Ac$_2$ to its parent molecule (Neu5Ac), along with a small amount of the anhydro compound in each case (data not shown). However, the amount of this anhydro compound was not increased by prior base treatment, suggesting that it only arose by acid catalysis. These results indicate that base-catalyzed formation of the 4,8-anhydro compound seen with free 4-*O*-acetylated sialic acids is not favored on the corresponding bound sialic acid.

Thus, there are at present no adequate conditions to obtain complete de-*O*-acetylation of free 4-*O*-acetylated sialic acids, to yield the parent compounds. On the other hand, glycosidically bound 4-*O*-acetylated sialic acids can be adequately de-*O*-acetylated using conventional conditions.

CONCLUSIONS

We have shown here that FAB-MS is not only the best initial screening approach for defining the complexity of mixtures of sialic acids but is also the best choice for the identification of the components, particularly when the amount of material is limited. The ability to study mixtures becomes especially important when analyzing biological materials whose composition is not known and which could contain one or more labile groups on the sialic acids. The periodate oxidation technique is then used to differentiate between different positional isomers. When working with a mixture, it is possible to carry out mild periodate treatment to oxidize the more reactive components (removing aliquots for analysis) and to then increase the temperature or the time of reaction to effect cleavage of the more resistant components.

The sensitivity of FAB-MS for the analysis of sialic acids is documented in this study. If the sample is clean, it is possible to detect as little as 0.1 μg (200–300 pmol) of a single sialic acid after derivatization. When analyzing a biologically derived sample that cannot be submitted to extensive purification because of the total amount available, it is still possible to recognize the peaks working at the low microgram range, although the chemical noise at every mass unit is higher than that obtained for highly purified compounds. For the confident detection of potentially novel substances, it is necessary to have a few micrograms as the minimum sample size prior to derivatization since it is best to carry out at least two experiments with different derivatives. The sensitivity is also improved by a proper handling of the sample and the complete elimination of salts. By combining appropriate derivatization procedures (carried out in one vial, without any rigorous purification involved), it is possible to unambiguously identify and roughly quantify even minor components (~0.5%) present in complex mixtures.

Special mention must be made of the problems involved in analyzing 7-*O*-acetylated sialic acids. It is well known that *O*-acetyl groups at the 7-position can migrate to the thermodynamically favored 9-positive under mildly alkaline conditions

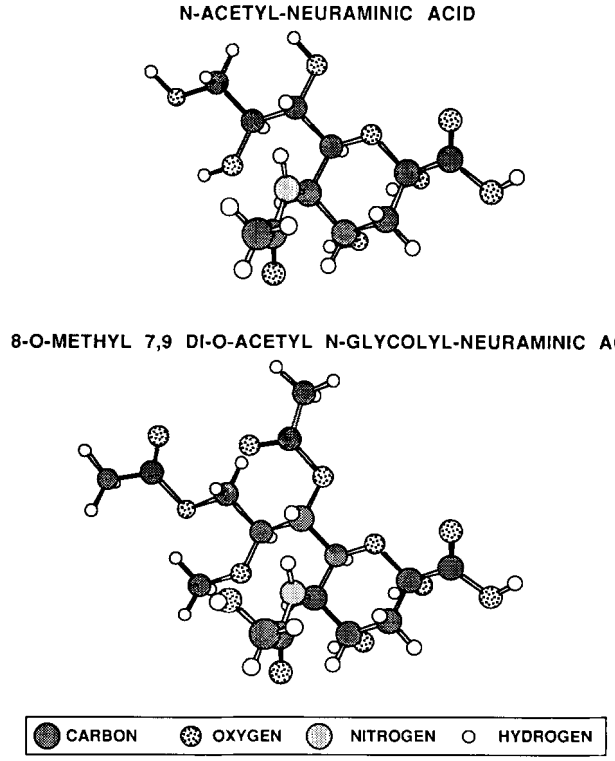

FIG. 20. **Structure of the new sialic acid from the starfish *P. brevispinus* in comparison to the parent molecule.**

(18, 45). Thus, if care is not exercised samples containing such molecules can give rise to a mixture of 7- and 9-*O*-acetylated compounds during isolation, purification, or subsequent handling. A further problem arises because periodate-induced loss of a 1-carbon unit from Neu5,7Ac$_2$ produces a molecule whose derivatives give exactly the same molecular ions as those obtained after loss of two carbons from Neu5Gc. Thus, we found that when starting with a mixture of Neu5,7Ac$_2$ and Neu5Gc7Ac, partial de-*O*-acetylation could yield the corresponding parent molecules, Neu5Ac and Neu5Gc, and prevent complete interpretation of the spectra. In this instance, it was necessary to prove the identity of the derivatized periodate oxidation products by subsequent deuteropermethylation.

Several examples have been presented to demonstrate the sensitivity of this approach for identifying sialic acids purified from biological sources. In some cases, samples thought to be pure standards were found by FAB-MS to contain trace amounts of other sialic acids that were not detected by the conventional HPLC and TLC methods. In other cases, minor components previously unknown in certain biological samples were detected. In the case of alkaline de-*O*-acetylation of sialic acids, we found the major product of de-*O*-acetylation of 4-*O*-acetylated sialic acids was not the parent compound, but rather 4,8-anhydroneuraminic acid which had previously been described from other sources (43, 44). In fact, this study shows that complete de-*O*-acetylation of free 4-*O*-acetyl sialic acids without generation of the anhydro compound is not possible using simple alkaline hydrolysis. In contrast, we show that glycosidically bound 4-*O*-acetylated sialic acids are not able to generate the anhydro compound upon alkaline de-*O*-acetylation, and therefore can be completely de-*O*-acetylated. Given the extreme lability of the 4-*O*-acetyl group to acid hydrolysis, and the complete resistance of 4-*O*-acetylated sialic acids to all neuraminidases, it appears that current technology does not provide ways to quantitatively study this class of sialic acids.

For the confident detection of new sialic acids, the combination of the direct FAB-mass spectrum of the native molecule, and the FAB spectra after two different derivatization procedures is necessary. The periodate oxidation technique is then used to differentiate between different possible positional isomers. This overall strategy was employed to analyze the sialic acids from the polar lipids of the starfish *P. brevispinus*, in which we identified a new sialic acid, 8-*O*-methyl-7,9-di-*O*-acetyl-*N*-glycolylneuraminic acid. A model of this compound is shown in Fig. 20, in comparison with the common sialic acid, *N*-acetylneuraminic acid. It can be seen that the substitutions substantially change the overall size, structure, and shape of the molecule. It would not be surprising therefore, if this modified sialic acid has important biological roles in the organism.

Thus, we believe that FAB-MS with appropriate derivatization procedures, and the use of controlled periodate degradation, is the approach of choice for the identification of the various components in mixtures of sialic acids obtained from biological sources.

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SUPPLEMENTAL MATERIAL TO:

STUDIES OF NATURALLY OCCURRING MODIFICATIONS OF SIALIC ACIDS BY FAST-ATOM BOMBARDMENT-MASS SPECTROMETRY: ANALYSIS OF POSITIONAL ISOMERS BY PERIODATE CLEAVAGE

by

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Materials: The following materials were obtained from the sources indicated: Bovine submaxillary glands, Pel Freeze Biologicals; equine serum, Gibco Laboratories; *Pisaster brevispinus* (starfish), Scripps Institute of Oceanography, La Jolla, CA; Cellulose MN 2100 ff, Macherey, Nagel & Co., Duren, Germany; and pre-coated cellulose plates, .1 mm thickness, (10 x 20 cm), Merck. Dowex 50 AG 1x8 (hydrogen form) from Biorad was washed extensively with water prior to use. Dowex-3x4A (chloride form), from Biorad was converted to the formate form, and equilibrated in 10mM sodium formate, pH 5.5. All other chemicals were of reagent grade and were purchased from commercial sources.

Sources and purification of sialic acids: N-acetylneuraminic acid (Neu5Ac) (>99% purity) was from Kantoishi Pharmaceutical Co., Tokyo, Japan; other sialic acids were isolated and purified from biological sources as follows:

Sialic acids from Bovine submaxillary mucin(BSM). 1.0 kg of bovine submaxillary glands was dissected free of the surrounding fats and nodes, finely minced and homogenized in 5 volumes of water in a Waring Blender. The mucin was purified in a manner similar to that described by Nisizawa and Pigman (29). Briefly, after centrifugation at 7,000 RPM for 30 min at 4°C, the supernate was filtered through a glass wool plug to remove macroscopic lipid material, and the pellet was re-extracted twice in a similar fashion. The mucin was precipitated by gradual acidification to pH 3.5 with 50 mM HCl at 4°C, collected by centrifugation at 1,500 RPM for 15 min at 4°C, washed with cold water and resuspended in 5 vol. of water. The mucin was dissolved by gradually neutralizing to pH 7.5 with dropwise addition of 50mM NaOH at 4°C. After 24 hrs of dialysis against water at 4°C, the BSM was recovered by lyophylization (yield = 15.23 g). The BSM was suspended in 2M acetic acid (10 g/350 ml) and incubated at 80°C for 3 hrs with constant stirring to release the sialic acids with minimal loss of O-acetyl groups (18). In other preparations, the mucin was hydrolyzed directly after precipitation as described by Schauer and others (21). After hydrolysis and centrifugation at 7,500 RPM for 30 min, the solution was dialysed (1,000 MW cut-off tubing) against 10 vol. of water at 4°C for 24 hrs, and the dialysate lyophylized (yield = 2.15 g). The mixture of sialic acids was purified by sequential ion exchange chromatography as previously described (18). Briefly, 2 g of sialic acids dissolved in 500 ml of water were loaded on a Dowex-50 AG (hydrogen form) cation exchange column (500 ml). The column was washed with 2 L of water and this effluent (pH was taken to 3.5 by addition of 10 mM sodium formate pH 5.5) was immediately loaded on a Dowex -3X4A (formate form) anion exchange column, which was washed with 2 L of 10 mM formic acid, and eluted with 5 L of 1M formic acid (yield = 653 mg). Further purification and separation of sialic acid species was achieved by chromatography on Cellulose MN 2100 ff Macherey (600 ml column, 40 mg of sialic acids loaded each time) essentially as described by Schauer et al. (21,30). Each fraction was analyzed by the 2-thiobarbituric acid (TBA) assay (31) before and after base treatment (18) with the modifications described below. The composition of each of the TBA-positive fraction from the cellulose column (250 x 2.5 ml fractions) was analyzed by Thin Layer Chromatography (TLC) on cellulose, and every other fraction was also analyzed by HPLC on a Aminex HPX-72 S column as described below. The HPLC eluant was monitored by absorbance at 200nm, in comparison with previously known standards (26). Those fractions that showed more than one peak were submitted to preparative HPLC in the same system. Each peak was pooled, and processed as described below. In some cases, two or three successive preparative HPLCs were necessary in order to obtain pure compounds. Typical final yields of pure sialic acids from 40 mg of the mixture of purified sialic acids were: Neu5,7,8,9Ac4, 50 nmoles; Neu5,7(8),9Ac3: 250 nmoles, Neu5,9Ac2, 6 umoles; Neu5,7Ac2, 6 umoles; Neu5Ac, 50 umoles, and Neu5Gc: 45 umoles.

Sialic acids from equine serum (ES). 450 ml of equine serum was dialysed for 24 hrs against water using a dialysis tubing with a 12,000 MW cut-off, and the pH adjusted to 2.0 (30) by dropwise addition of concentrated formic acid. The solution was heated at 70° for 1 hr, centrifuged, and the supernatant dialyzed overnight against 10 vol. of water (1,000 MW cut-off tubing) at 4°C and lyophylized. The residue was retreated twice under the same conditions. The acid hydrolysis conditions are different from those used for release of sialic acids from BSM because, in this case, it was necessary to protect the labile 4-O-acetyl groups (32). The lyophylized difusates were pooled and the mixture of sialic acids purified by ion exchange and cellulose chromatography as described above for BSM. The fractions from the cellulose column were analysed by the TBA reaction (before and after base treatment), by thin layer chromatography on cellulose plates (before and after exposure to ammonia vapors), and every fourth fraction was analyzed by FAB-MS.

Sialic acids from the starfish *Pisaster brevispinus*. 3 animals (2.3 kg) were finely minced and total lipids extracted into chloroform/methanol mixtures (2:1, 1:1, and 1:2, v/v). The pooled extracts were dried down using a rotary evaporator, dissolved in chloroform:methanol, 2:1 (v/v), and submitted to Folch partition (33). The polar lipids from the upper layer of the Folch partition were recovered, after dialysis against water, by lyophylization (yield = 2.2 g). The sialic acids present in this lipid extract (27.7 nmoles/mg as determined by the TBA assay after base treatment) were released by acid hydrolysis. A portion of the lipids (22.3 mg) were treated with 3.1 ml of 2M acetic acid (in the presence of 1% BHT to inhibit lipid peroxidation) for 5h at 80°C (this period of the treatment was shown to cause maximum release). The suspension was dialysed for 24 hr at 4°C against 20 vol. of water (1,000 MW cut-off tubing), and the diffusate containing the sialic acids was directly loaded on a Dowex-50 (hydrogen form) column (10 ml). The column was washed with water (100 ml) and the effluent lyophylized (yield = 315 nmoles), dissolved in water and loaded on a Dowex-3x4A (formate form) column (1 ml). The sialic acids were eluted with 1 M formic acid, and recovered by lyophylization (yield = 218 nmoles). These sialic acids were analyzed as a mixture without further fractionation.

Analysis of sialic acids: The 2-thiobarbituric acid (TBA) assay was used to assay free sialic acids (31). For bound sialic acids a previous hydrolysis with 0.1N sulfuric acid for 1 hr at 80° was usually performed. Analytical de-O-acetylation was always carried out to quantify the interference caused by the presence of O-acetyl substituents at the 7-, 8-, or 9- positions. The samples were treated with 0.1 M NaOH for 30 min at 37°C, and the reaction was quenched by addition of 0.2M HCl (5ul for10 ul of reaction mixture) (26). In the case of 4-O-acetylated sialic acids, we found that these conditions were not suitable for de-O-acetylation (see "Results and Discussion").

Thin layer chromatography: Sialic acids were analysed by thin layer chromatography on precoated cellulose plates using n-butanol:n-propanol:0.1 M HCl, 1:2:1 (v/v/v) as developing solvent. The periodate/resorcinol reagent, prepared as described by Jourdian et al. (34) was used for staining. When monitoring the cellulose chromatography of sialic acids from equine serum, the TLC was done directly and also after spotting the samples, exposing the plates overnight to ammonia vapors.

High performance liquid chromatography (HPLC): Analytical and preparative chromatography of sialic acid mixtures were performed on a Biorad HPX-72-S (0.78 cm x 30 cm) eluted in the isocratic mode with 100 mM Na_2SO_4 at 1 ml per min (26). The elution was followed by monitoring the absorbance at 200 nm. In the case of preparative runs the peaks were pooled at the outlet of the UV detector according to the absorbance reading, and immediately placed on ice. The pooled products were diluted 20 times with water and immediately loaded on a Dowex-3x4A (formate form) column maintained at 4°C. After washing with 7 volumes of 10 mM formic acid, the sialic acids were eluted with 1 M formic acid, and recovered by lyophylization.

Fast-atom bombardment mass spectrometry: The purified sialic acids were submitted to FAB-MS in both negative and positive ion modes (27). Native samples were dissolved in 5% aqueous acetic acid (0.1-5 ug/ul) and 1 ul was loaded into glycerol or glycerol/thioglycerol (1:1) on the FAB target. Derivatized samples were extracted as described below, and then loaded. Analysis was carried out using a VG Analytical ZAB-HF mass spectrometer fitted with an M-Scan FAB gun operated at 8 KV. Xenon was used as the bombarding gas. Spectra were acquired on oscillographic chart paper and were manually counted.

Preparation of sialic acid derivatives: Water (10 ul) was added to the dried sialic acid (1 ug) in a screw-capped tube and the reducing agent (20 ul of a solution prepared by mixing 350 ul of methanol, 41 ul of acetic acid and 3 mg of sodium cyanoborohydride) was added, and the resulting solution incubated at 80°C for 1 hr. The reaction was stopped with water (1 ml) and the excess reagents removed by ether extraction (5x1ml). The aqueous layer was dried, the acylating reagent (200 ul) was added and the solution was left at room temperature for 10 min . The reagents were then removed under a stream of N_2. The residue was dissolved in chloroform (500 ul) and washed with water (3x1 ml). The chloroform solution was dried and the sample re-dissolved in methanol prior to FAB-MS. The acylating reagents were prepared as follows: trifluoroacetic anhydride and the appropriate acid (acetic acid, deuteroacetic acid, or propionic acid) were mixed in an equimolar ratio and left for a few minutes to cool prior to adding the mixture to the sample. The permethylated (and deuteromethylated) derivatives were prepared by the procedure of Ciacanu and Kerek (35) as follows: a slurry of NaOH/DMSO was prepared by grinding eight pellets of NaOH in 2ml of DMSO using a mortar and pestle. The resulting suspension (0.5ml) was added to the dried sample followed by methyl (or deuteromethyl) iodide (0.25ml), and the mixture shaken at RT for 10min. The reaction was quenched with water (1ml), the product extracted into chloroform (1ml), washed with water (4 x 1 ml) and dried under a stream of nitrogen. The sample was dissolved in methanol (10 ul) prior to FAB analysis.

Periodate oxidation: A solution of sodium metaperiodate in water (10mM, 20 ul, pH adjusted to 5.5 by 5% acetic acid, or buffered with 10 mM sodium acetate pH 5.5) was added to the dried sialic acid (10-20 nmol) sample and incubated in the dark at 0°C for 10 min followed by 3 hr at R.T. Aliquots (5 ul) were removed after 10 min, 70 min and 190 min. Each aliquot was quenched by adding ethylene glycol (10ul) followed by water (10 ul) and 25 ul of the ABEE reagent (prepared by dissolving 3.5 mg of ABEE, 0.5 mg of sodium cyanoborohydride in 350 ul of methanol, and 40 ul of glacial acetic acid). Each reaction was incubated at 80°C for 1 hour, quenched with water (1 ml), extracted with ether (4x1ml) and the water layer was lyophylized. The dried residue was peracylated by adding 200ul of TFAA: RCO_2H (2:1, v:v, R= $-CH_3$, $-CD_3$, $-CH_2CH_3$) and leaving the mixture at room temperature for 10 min (36). The reagents were removed under a stream of nitrogen and the products were taken up in $CHCl_3$ (1 ml) and washed with water (3x1ml). The $CHCl_3$ was removed under a stream of nitrogen and the sample redissolved in methanol (5-10ul) for subsequent FAB-MS analysis.

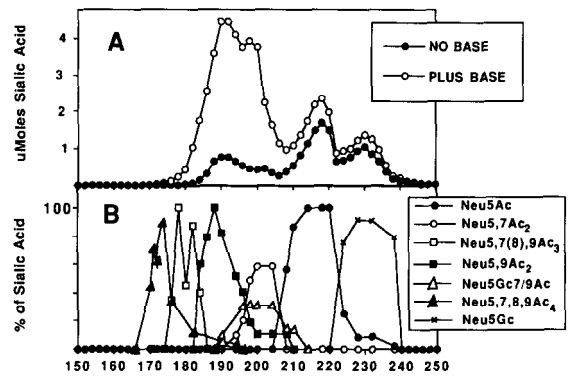


Figure 2. Example of purification of sialic acids by sequential cellulose chromatography and analytical HPLC of selected fractions. Sialic acids from BSM were released, purified and fractionated on a cellulose column as described under "Experimental Procedures". Every other fraction was monitored by the TBA assay before and after de-O-acetylation (Panel A) and by HPLC (Panel B) as described under "Experimental Procedures".

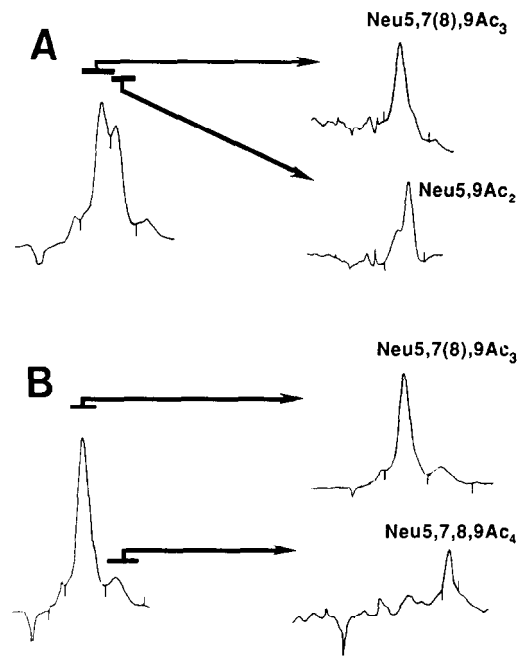


Figure 3. Examples of Preparative HPLC of fractions from cellulose chromatography. Fractions from the cellulose chromatography shown in Figure 2 were pooled and further separated by HPLC as described under "Experimental Procedures". The two examples shown are A (Fractions 178-182): separation of mono- and di-O-acetylated sialic acids; and B(Fractions 170-177): separation of di- and tri-O-acetylated sialic acids.

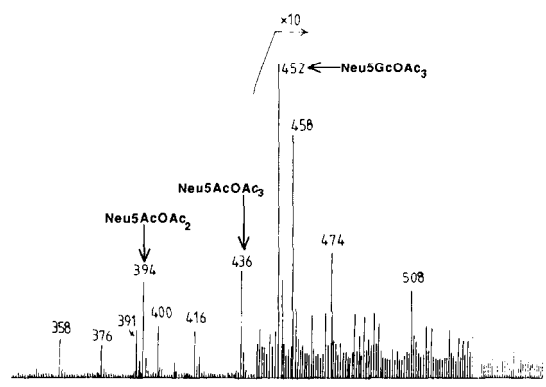


Figure 6. Native FAB spectrum of a mixture of native sialic acids from bovine submaxillary mucin. Sialic acids from BSM were released, purified and fractionated on a cellulose column as described under "Experimental Procedures". A fraction containing several different sialic acids (by HPLC analysis) was analyzed by FAB-MS in the positive ion mode.

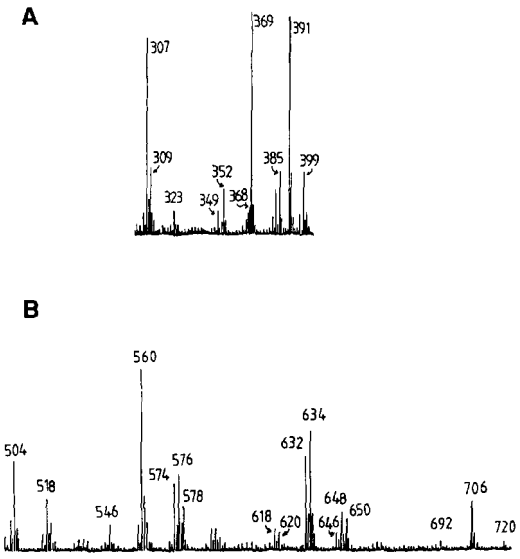


Figure 8. FAB spectra for Fraction containing Neu5,9Ac2. A sample containing Neu5,9Ac2 as the only known component was analyzed by FAB-MS either in the native form (negative mode, panel A), or as the reduced and perpropionylated derivative (positive mode, panel B).

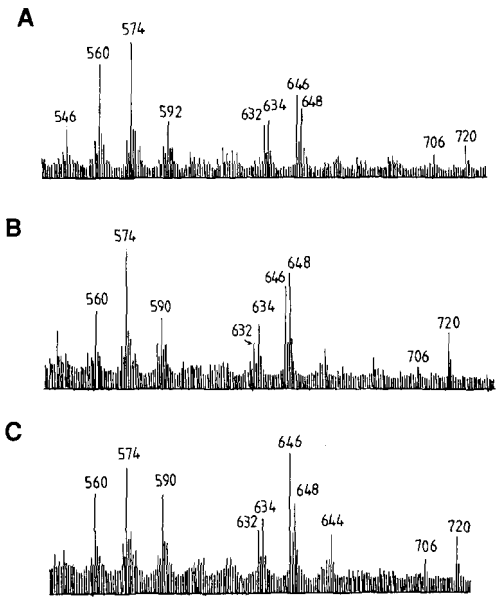


Figure 11. Positive FAB spectrum of mixtures of standard Neu5Ac, Neu5,9Ac2, and Neu5Gc of known composition: The three sialic acids standards were mixed, reduced, perpropionylated and loaded onto the FAB target. Panel A: ratio 1.0:1.0:1.0; Panel B: ratio 1.0:0.4:1.0, and Panel C: ratio 1.0:1.0:2.5.

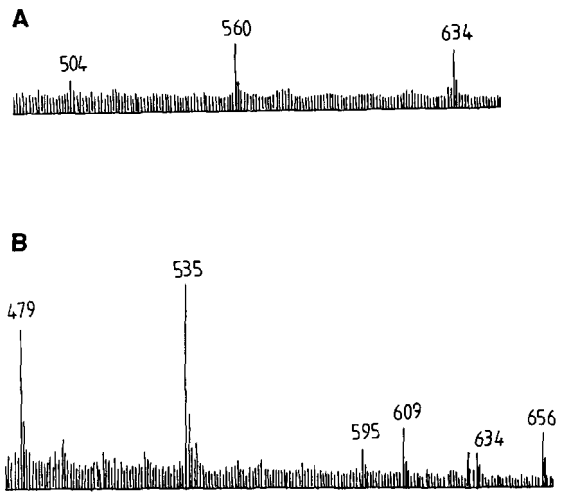


Figure 13. Positive FAB mass spectra of mono-O-acetylated sialic acids after mild periodate treatment, ABEE-tagging and perpropionylation. Samples of Neu5,9Ac2 (Panel A), and Neu5Ac4Ac (containing some Neu5Gc4Ac) (Panel B) were treated with periodate for 10 min on ice, and aliquots removed for ABEE-tagging and perpropionylation.

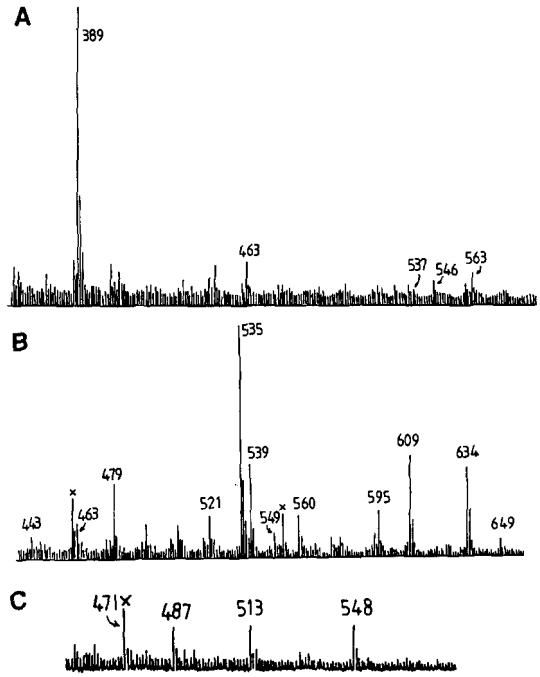
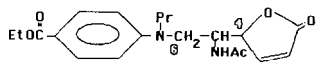
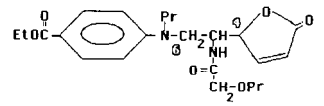
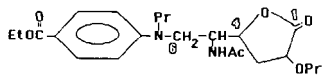
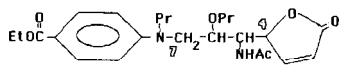
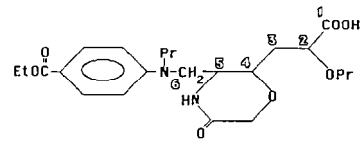
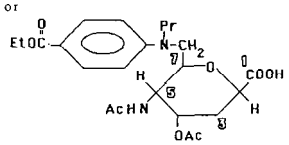
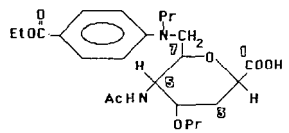
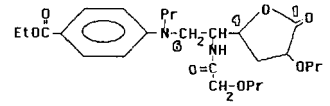
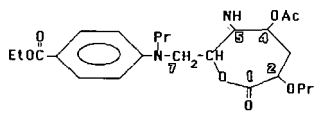
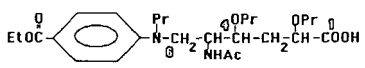
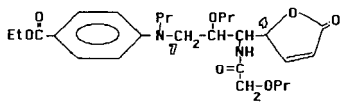
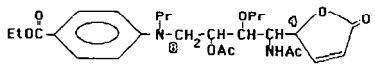
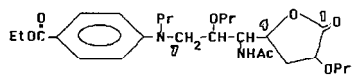
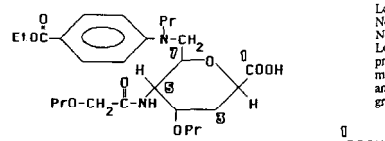
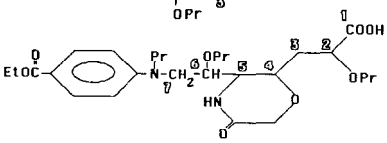
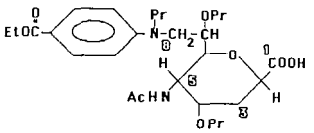
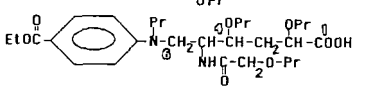
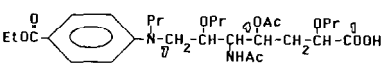
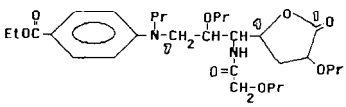
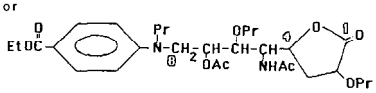
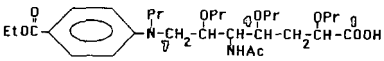
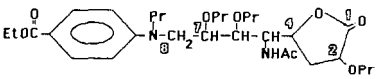
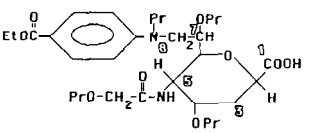
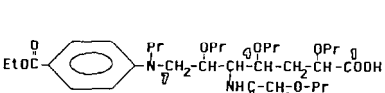
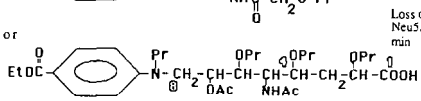
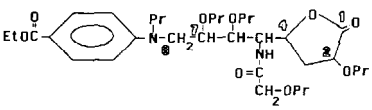
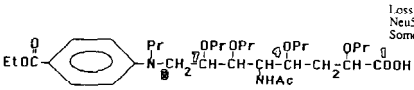
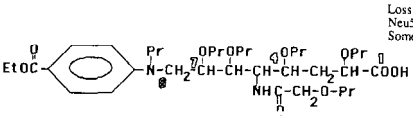


Figure 14. Positive FAB mass spectra of mono-O-acetylated sialic acids after strong periodate treatment, ABEE-tagging and perpropionylation. Samples of Neu5,9Ac2 (Panel A), and Neu5Ac4Ac/Neu5Gc4Ac (Panel B), were treated with periodate and aliquots removed for ABEE-tagging and perpropionylation after 3h at RT. Neu5,7Ac2 was treated with periodate for 3 hours, ABEE-tagged, reduced, perpropionylated, and then deuteropermethylated (Panel C).

TABLE 3

Proposed structures of perpropionylated fragments of various sialic acids following periodate treatment and ABEE-tagging under reducing conditions. [The numbers on the structures indicate the original numbers of the carbons (1-9) in the sialic acid molecules; Pr= O-Propionyl group.]

m / z	Proposed Structure	Comments
389		Loss of C-7/8/9 from: Neu5Ac in 10 min Neu5,9Ac2 in 3h Loss of two propionic acid molecules (76 m.u. each) Major component
461		Loss of C-7/8/9 from: Neu5Gc in 3h Neu5Gc9Ac in 3h Neu5Gc7,9Ac2 in 3h
463		Loss of C-7/8/9 from: Neu5Ac in 3h Neu5,9Ac2 in 3h Loss of one propionyl group (56 m.u.) Minor component
475		Loss of C-8/9 from: Neu5Ac in 10 min Neu5,9Ac 2 in 3h Loss of two propionic acid molecules (74 m.u. each) Sometimes absent
479		Loss of C-7/8/9 from: Neu5Gc in 3h Neu5Gc9Ac in 3h Neu5Gc7,9Ac2 in 3h Loss of a propionic acid molecule (74 m.u.) and a propionyl group (56 m.u.)
	or	
		Loss of C-8/9 from: Neu4,5Ac2 in10 min Loss of one propionic acid molecule (74 m.u.) and one propionyl group (56 m.u.) Major component
493		Loss of C-8/9 from: Neu5Ac in 10 min Neu5,9Ac2 in 3h Loss of one propionic acid molecule (74 m.u.) and one propionyl group (56 m.u.) Major component
535		Loss of C-7/8/9 from: Neu5Gc in 3h Neu5Gc9Ac2 in 3h Neu5Gc7,9Ac2 in 3h Loss of a propionic acid molecule (74 m.u.)
	or	
		Loss of C-8/9 from: Neu4,5Ac2 in 10 min Loss of a propionic acid molecule (74 m.u.) Major component
537		Loss of C 7/8/9 from: Neu5Ac in 3h Neu5,9Ac2 in 3h Minor component
547		Loss of C 8/9 from: Neu5Gc in 10 min Neu5Gc9Ac in 3h Loss of two propionic acid molecules (74 m.u. each)
	or	
		Loss of C 9 from: Neu5,7Ac2 in 10min Loss of two propionic acid molecules (74 m.u. each)
549		Loss of C-8/9 from: Neu5Ac in 10 min Neu5,9Ac2 in 3h Loss of a propionic acid molecule (74 m.u.) Major component
565		Loss of C-8/9 from: Neu5Gc in 10 min Neu5Gc9Ac in 3h Loss of one propionic acid molecule (74 m.u.) and one propionyl group (56 m.u.)
		
579		Loss of C-9 from: Neu5Ac in 10 min Loss of two propionic acid molecules (74 m.u. each) Sometimes absent
609		Loss of C-7/8/9 from: Neu5Gc in 3 h N3u5Gc9Ac in 3h Neu5Gc7,9Ac2 in 3h
	or	
		Loss of C 8/9 from: Neu4.5Ac2 in 10min Minor component
621		Loss of C-8/9 from: Neu5Gc in 10 min Neu5Gc9Ac in 3h Loss of one propionic acid molecule (74 m.u.)
	or	
		Loss of C 9 from: Neu5,7Ac2 in 10min Loss of one propionic acid molecule (74 m.u.)
623		Loss of C-8/9 from: Neu5Ac in 10 min Neu5,9Ac2 in 3h Minor component
635		Loss of C-9 from: Neu5Ac in 10 min Loss of one propionic acid molecule (74 m.u.) Sometimes absent
651		Loss of C-9 from: Neu5Gc in 10 min Loss of one propionic acid molecule (74 m.u.) and one propionyl group (56 m.u.) Sometimes absent
695		Loss of C-8/9 from: Neu5Gc in 10 min Neu5Gc9Ac in 3h
	or	
		Loss of C 9 from: Neu5,7Ac2 in 10 min
707		Loss of C-9 from: Neu5Gc in 10 min loss of one propionic acid molecule (74 m.u.)
709		Loss of C-9 from: Neu5Ac in 10 min Sometimes absent
781		Loss of C-9 from: Neu5Gc in 10 min Sometimes absent