Eur. J. Biochem. 134, 139 – 143 (1983)
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Identification of New Sialic Acids Derived from Glycoprotein of Bovine Submandibular Gland

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(Received March 10, 1983) – EJB 83 0230

Improvements in the isolation procedure and the analytical equipment enabled the detection of seven novel sialic acids in bovine submandibular gland glycoprotein: *N*-acetyl-8-*O*-acetylneuraminic acid, *N*-acetyl-8,9-di-*O*-acetylneuraminic acid, *N*-acetyl-7,8,9-tri-*O*-acetylneuraminic acid, *N*-glycoloyl-7-*O*-acetylneuraminic acid, *N*-glycoloyl-7,9-di-*O*-acetylneuraminic acid, *N*-glycoloyl-8,9-di-*O*-acetylneuraminic acid, and *N*-glycoloyl-7,8,9-tri-*O*-acetylneuraminic acid. There are also indications for the presence of *N*-glycoloyl-8-*O*-acetylneuraminic acid. In addition, the sialic acids already known to occur in this tissue, namely *N*-acetylneuraminic acid, *N*-acetyl-7-*O*-acetylneuraminic acid, *N*-acetyl-9-*O*-acetylneuraminic acid, *N*-acetyl-7,9-di-*O*-acetylneuraminic acid, *N*-glycoloylneuraminic acid, and *N*-glycoloyl-9-*O*-acetylneuraminic acid could be identified.

Sialic acids were released from the mucin by mild acid hydrolysis and prefractionated on a Dowex 2X8 anion-exchange column (formate form) by elution with a 0 – 0.6 M gradient of formic acid. The four pools of sialic acids obtained in this way were each further fractionated by column chromatography on cellulose with *n*-butanol/*n*-propanol/water (1/2/1, v/v/v) as eluent. By this procedure, *N*-acetylneuraminic acid, *N*-acetyl-7-*O*-acetylneuraminic acid, *N*-acetyl-9-*O*-acetylneuraminic acid, *N*-glycoloylneuraminic acid, and *N*-glycoloyl-9-*O*-acetylneuraminic acid were obtained in pure form. The other sialic acids could be enriched sufficiently in different fractions for structural identification. Analyses of sialic acids were carried out by one-dimensional and two-dimensional thin-layer chromatography, gas-liquid chromatography, and gas-liquid chromatography – mass spectrometry.

Sialic acids are known to form an integral part of gangliosides, many glycoproteins and oligosaccharides. Of the *O*-acylated species, the 9-*O*-acetylated *N*-acetyl- and *N*-glycoloylneuraminic acids are by far the most predominant [1]. The greatest variety of sialic acids in the biological materials analyzed so far has been found in bovine submandibular gland glycoprotein. The presence of Neu5Ac, $Neu5,7Ac_2$, $Neu5,9Ac_2$, $Neu5,7,9Ac_3$, Neu5Ac9Lc, Neu5Gc, and Neu-9Ac5Gc in this tissue has earlier been established unequivocally by GLC and GLC-MS [2]. Analysis of crude sialic acid preparations by TLC and 360-MHz ^{1}H-NMR spectroscopy (unpublished results) gave indications for the occurrence of still other sialic acids in bovine submandibular gland.

Abbreviations. Neu5Ac, *N*-acetylneuraminic acid; $Neu5,7Ac_2$, *N*-acetyl-7-*O*-acetylneuraminic acid; $Neu5,8Ac_2$, *N*-acetyl-8-*O*-acetylneuraminic acid; $Neu5,9Ac_2$, *N*-acetyl-9-*O*-acetylneuraminic acid; $Neu5,7,9Ac_3$, *N*-acetyl-7,9-di-*O*-acetylneuraminic acid; $Neu5,8,9Ac_3$, *N*-acetyl-8,9-di-*O*-acetylneuraminic acid; $Neu5,7,8,9Ac_4$, *N*-acetyl-7,8,9-tri-*O*-acetylneuraminic acid; Neu5Ac9Lc, *N*-acetyl-9-*O*-L-lactoylneuraminic acid; Neu5Gc, *N*-glycoloylneuraminic acid; Neu7Ac5Gc, *N*-glycoloyl-7-*O*-acetylneuraminic acid; Neu8Ac5Gc, *N*-glycoloyl-8-*O*-acetylneuraminic acid; Neu9Ac5Gc, *N*-glycoloyl-9-*O*-acetylneuraminic acid; $Neu7,9Ac_25Gc$, *N*-glycoloyl-7,9-di-*O*-acetylneuraminic acid; $Neu8,9Ac_25Gc$, *N*-glycoloyl-8,9-di-*O*-acetylneuraminic acid; $Neu7,8,9Ac_35Gc$, *N*-glycoloyl-7,8,9-tri-*O*-acetylneuraminic acid; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; GLC-MS, gas-liquid chromatography – mass spectrometry; The abbreviations for sialic acids conform to the proposals for the nomenclature of natural sialic acids drawn up on the basis of a discussion during the 5th International Symposium on Glycoconjugates held at Kiel in 1979.

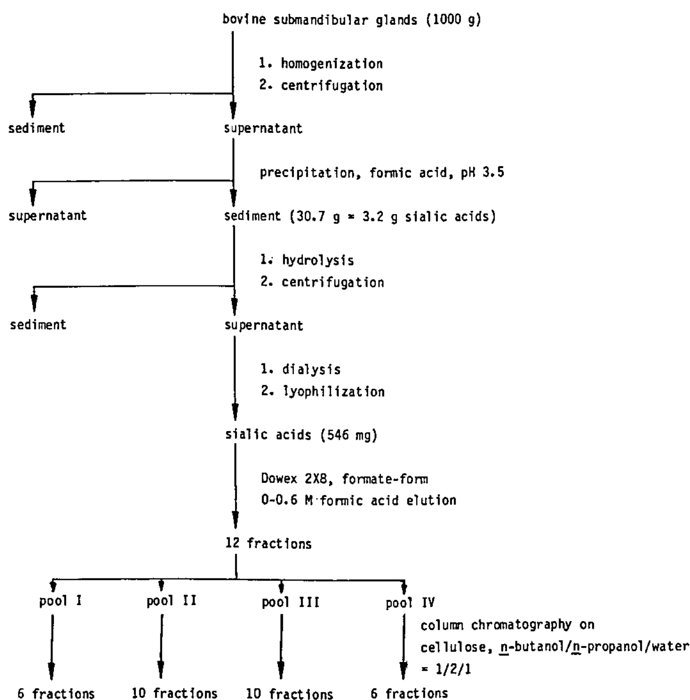
The aim of the present study was to establish the nature of these unknown sialic acids and to develop a system for isolation and analysis, which is generally useful and applicable for the identification of these compounds. The elucidation of the complete pattern of sialic acids is essential for the understanding of the biological significance of the species-specific and tissue-specific diversity of sialic acids and especially of the occurrence of *O*-acyl groups in these compounds. Finally, knowledge of the position of the *O*-acyl groups on sialic acids is a prerequisite for the study and classification of special enzymes involved in the biosynthesis of *N*,*O*-acylated sialic acids.

MATERIALS AND METHODS

Isolation of Sialic Acids

The isolation procedure of sialic acids was in principle based on methods described in [3]. Unless otherwise stated all procedures were carried out at 2 – 4 °C. A flow diagram of the different isolation steps is given in Scheme 1.

Preparation of Glycoprotein. Bovine submandibular glands were acquired in the local slaughter house immediately after death of the animals and cooled on ice. Fat and connective tissue were removed, the glands (1000 g) were frozen, tri-



Scheme 1. *Isolation procedure for sialic acids from bovine submandibular glands*

turated, taken up in 3.5 l of water, homogenized with an Ultraturrax (Janke & Kunkel, Staufen), and centrifuged for 30 min at $6000 \times g$. The sediment was resuspended in 1 l of water, homogenized and centrifuged under the same conditions. The combined supernatants were adjusted to pH 3.5 with formic acid to precipitate the major amount of mucous glycoprotein, and subsequently centrifuged for 30 min at $6000 \times g$. The supernatant containing 10 – 30% of the total sialic acids was discarded and the sediment lyophilized. The yield was 30.7 g, containing 3.2 g of sialic acids as estimated with the orcinol/Fe^{3+}/HCl reagent [4].

Acid Hydrolysis. A suspension of the glycoprotein (30.7 g) in 1 l of water was adjusted to pH 2 with conc. formic acid, hydrolyzed for 1 h at 70 °C, cooled to room temperature, and centrifuged for 30 min at $6000 \times g$. The supernatant was filtered and then dialyzed in an Amicon DC-2 cell with a hollow fiber cartridge H1P10. The dialysate was lyophilized; it contained 546 mg of sialic acids.

Anion-Exchange Chromatography. The lyophilizate (546 mg) was dissolved in 3 l of water and passed through a column (50×3.5 cm) filled with Dowex 2X8 anion-exchange resin, 100 – 200 mesh, formate form (Fluka, Neu-Ulm). The resin was washed with 2 l of water and sialic acids were eluted with 4 l of a linear gradient from 0 – 0.6 M formic acid. Twelve sialic-acid-containing fractions of about 80 ml each, were collected and analyzed by TLC in one and two dimensions, by GLC and GLC-MS, as described below. The fractions with a similar pattern of sialic acids were combined to give four pools (I – IV) and lyophilized.

Column Chromatography on Cellulose. Portions of sialic acids (maximum 50 mg) from pools I – IV were dissolved in 1 – 2 ml of *n*-butanol/*n*-propanol/water (1/2/1, v/v/v). After centrifugation, each supernatant was applied to a column (150×3.5 cm) filled with cellulose (MN 2100ff, Macherey, Nagel & Co., Düren) equilibrated in the *n*-butanol/*n*-propanol/water mixture. Sialic acids were eluted with this solvent at a flow rate of about 8 ml/h using a peristaltic pump. Fractions of 4 – 5 ml were collected and tested with the orcinol/Fe^{3+}/HCl reagent. Sialic-acid-positive fractions were further investigated by TLC. Tubes containing the same sialic acid species or a similar pattern of different sialic acids were combined to give 6, 10, 10, and 6 fractions, respectively from the four pools I – IV. Each of these fractions was lyophilized and analyzed by GLC on SE-30 and in part on OV-17, as well as by GLC-MS.

Analysis of Sialic Acids

Colorimetry. Sialic acids were analyzed by the orcinol/Fe^{3+}/HCl reagent [4]. Crystalline Neu5Ac served as reference compound.

Thin-layer Chromatography. TLC was performed on cellulose sheets (Merck, Darmstadt) prerun in 0.1 M HCl, with *n*-butanol/*n*-propanol/0.1 M HCl (1/2/1, v/v/v) as solvent. For TLC in two dimensions [5] the same solvent was used for both directions, with an intermediate exposure of the TLC plate to NH_3 leading to de-*O*-acetylation (chamber containing 5 M NH_4OH, 2 h, room temperature). The sialic acid spots were visualized with the orcinol/Fe^{3+}/HCl spray reagent [3].

Gas-Liquid Chromatography. Lyophilized sialic acid samples (50 – 100 µg) were converted either into their trimethylsilyl esters, ethers by treatment with *N*-trimethylsilylimidazole [6] or into their methyl esters, trimethylsilyl ethers by treatment with diazomethane followed by hexamethyldisilazane/trimethylchlorosilane/pyridine [7]. GLC analysis of the trimethylsilyl esters, ethers was carried out on a Packard 428 gas chromatograph equipped with a flame-ionization detector and a glass column (220×0.2 cm) packed with 3.5% OV-17 on Chromosorb W/AW-DMCS, 80 – 100 mesh. The column oven temperature was programmed from 200 – 280 °C with a rate of 2 °C/min; the nitrogen flow-rate was 30 ml/min [6]. The methyl esters, trimethylsilyl ethers were analyzed on a Varian Aerograph 2740-30-01 equipped with dual flame-ionization detectors or on the Packard 428 gas chromatograph mentioned above; both systems were equipped with glass columns (200×0.4 cm) packed with 3.8% SE-30 on Chromosorb W/AW-DMCS, HP, 80 – 100 mesh. The column oven temperatures were 215 °C, the nitrogen flow-rates were 40 ml/min and 30 ml/min, respectively [7].

Gas-Liquid Chromatography – Mass Spectrometry. Combined GLC-MS of the methyl esters, trimethylsilyl ethers of sialic acids [2] was performed either on a Hewlett-Packard 5710 A gas chromatograph (glass column 200×0.4 cm; column material SE-30; column oven temperature 210 °C) combined with a Jeol JMS-D300 mass spectrometer (electron energy 70 eV; ion-source temperature 250 °C; accelerating voltage 1.5 kV; ionization current 300 µA) equipped with a Jeol JMA-2000 mass-data analysis system, or on a Varian 3700 gas chromatograph (glass column 200×0.2 cm; column material SE-30; column oven temperature 215 °C) coupled to a Varian MAT 44S mass spectrometer (electron energy 70 eV; ion-source temperature 220 °C; ionization current 300 µA) and a Varian Spectro Spin MAT 200 data processing system. Mass spectra of sialic acids are mainly interpreted on guidance of the characteristic fragment ions A – H, the formation of which is shown in Fig. 1. As was demonstrated earlier [2, 7] these fragments give information about the number, type, and

position of O-substituents and the type of N-substituents in sialic acids.

RESULTS AND DISCUSSION

A great variety of sialic acids was liberated by mild acid hydrolysis of bovine submandibular gland glycoprotein. Partial fractionation of this complex mixture was possible by Dowex 2X8 anion-exchange column chromatography (formate form) using a flat gradient of formic acid (0 – 0.6 M). In Fig. 2 the sialic acid pattern on TLC of the 12 fractions obtained is presented. Analysis of these fractions by GLC and GLC-MS revealed, besides of the presence of the already known sialic acids Neu5Ac, Neu5,7Ac_2, Neu5,9Ac_2, Neu-5,7,9Ac_3, Neu5Gc, and Neu9Ac5Gc [1,2], the novel compounds Neu5,8,9Ac_3 and Neu7,9$Ac_2$5Gc (see Fig. 2 and Tables 1 and 2). Fractions with a similar TLC pattern of sialic acids obtained from anion-exchange chromatography were combined to the pools I – IV (Fig. 2), and further fractionated by chromatography on cellulose. By this procedure 32 fractions were received in total (see Scheme 1) and analyzed by TLC, GLC and GLC-MS. As an example, the sialic acid pattern on TLC of the 10 fractions obtained from pool III is shown in Fig. 3. In addition to the sialic acids already identified after the purification by anion-exchange chromatography, five other unknown species were sufficiently enriched by column chromatography on cellulose in some fractions to enable identification by GLC-MS: Neu5,8Ac_2, Neu5,7,8,9-Ac_4, Neu7Ac5Gc, Neu8,9$Ac_2$5Gc, and Neu7,8,9$Ac_3$5Gc (see Tables 1 and 2). It has to be noted that Neu7Ac5Gc was present as a minor component in a fraction containing Neu5Gc and could not completely be separated from the latter compound by GLC on SE-30 (see Table 1). In another fraction, there were indications for the presence of Neu8Ac5Gc.

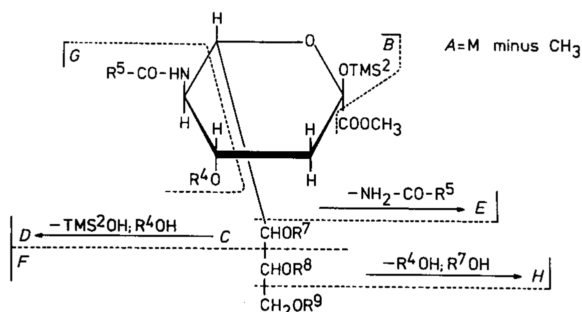


Fig. 1. *Formation of the characteristic fragment ions A – H used for the mass spectrometric analysis of sialic acid methyl esters, trimethylsilyl (TMS) ethers*

Applying the two fractionation procedures, the major sialic acids Neu5Ac, Neu5,7Ac_2, Neu5,9Ac_2, Neu5Gc, and Neu9Ac5Gc could be obtained in pure form. The amount of each of the newly identified sialic acids was estimated to be below 2% of the total sialic acid content in bovine submandibular gland glycoprotein.

Including the GLC-MS data obtained after column chromatography on cellulose of the pools I – IV, the elution behaviour of sialic acids from the anion-exchange resin can be recognized (Table 1). In general, Neu5Ac and its O-acetylated derivatives are eluted earlier than the corresponding Neu5Gc compounds. All Neu5Ac derivatives acetylated at O7, i.e. Neu5,7Ac_2, Neu5,7,9Ac_3, and Neu5,7,8,9Ac_4 are mainly eluted in the first three fractions (pool I), whereas species with an 8-O-acetyl group and without acetyl substitution at O7 are eluted later: Neu5,8Ac_2 in fractions 7 and 8 (pool III) and Neu5,8,9Ac_3 in fractions 8 – 12 (pools III and IV).

As can be seen from Table 1, the anomeric forms of the methyl esters, trimethylsilyl ethers of Neu5Ac, Neu5,7Ac_2, Neu5,7,9Ac_3, and Neu5Gc can partially be separated by GLC on SE-30. ^{1}H-NMR analysis of these sialic acids and other species in underivatized form [8] has shown that β-anomers are by far the predominant species. The anomeric assignment

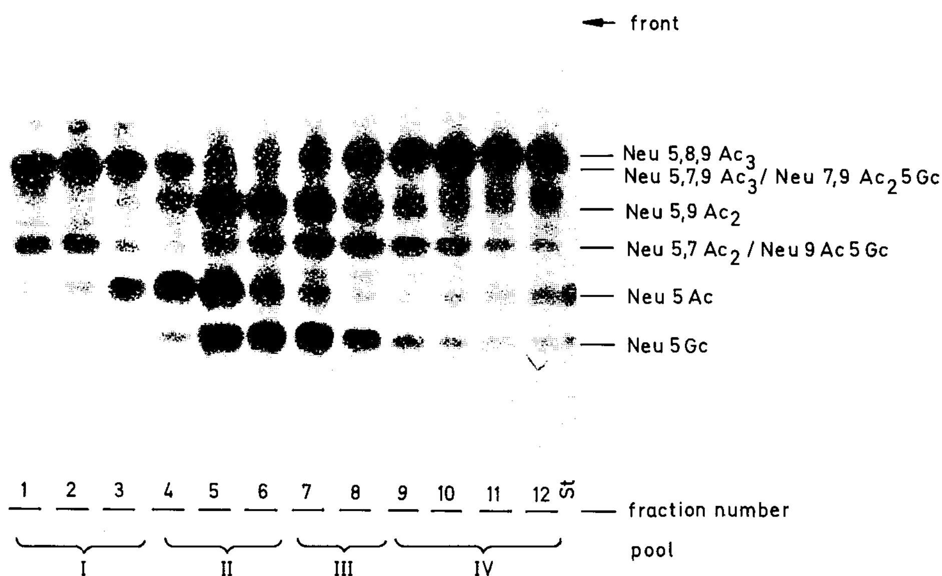


Fig. 2. *TLC of the different fractions containing sialic acids obtained by anion-exchange chromatography on Dowex 2X8.* Sialic acids were identified by GLC-MS. The combination of the fractions to give pools I – IV for column chromatography on cellulose is indicated. Reference Neu5Ac and Neu5Gc were run in the right lane (St)

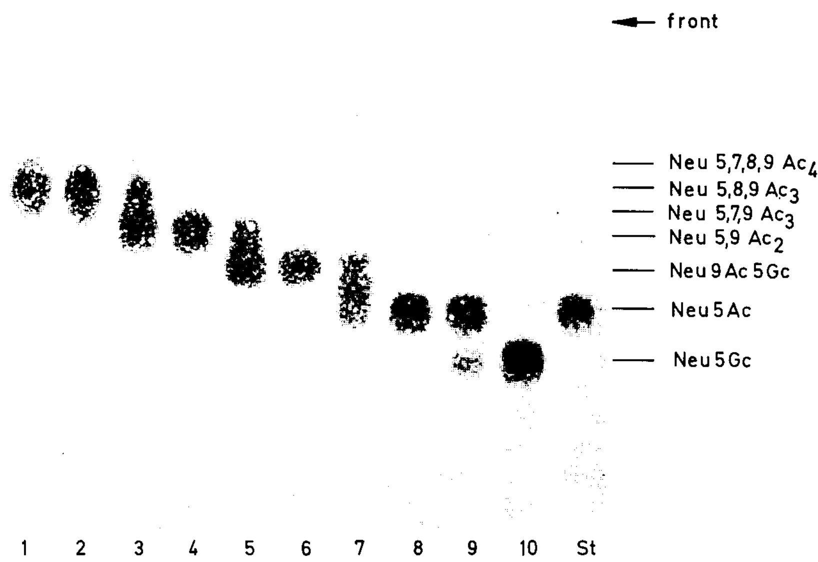


Fig. 3. *TLC of the ten different fractions containing sialic acids obtained from pool III by column chromatography on cellulose.* Sialic acids were identified by GLC-MS. Reference Neu5Ac was run in the right lane (St)

Table 1. *Elution behaviour on a Dowex 2X8 anion-exchange column (pools I–IV), TLC (R_F values) and GLC (R_{Neu5Ac} values) on SE-30 and OV-17 of sialic acids from bovine submandibular gland glycoprotein*
On SE-30, sialic acids were analyzed as methyl esters, trimethylsilyl ethers and identified by GLC-MS; on OV-17, sialic acids were analyzed as trimethylsilyl esters, ethers. For TLC and GLC on OV-17 sialic acids were identified by correlation with the results of GLC-MS on SE-30. *, new sialic acid; –, not determined

Compound	Pool	R_F value	R_{Neu5Ac} value	
			SE-30	OV-17
Neu5Ac	I–IV	0.45	β 1.00 α 1.05	1.00
Neu5,7Ac2	I	0.54	β 1.04 α 1.00	1.20
Neu5,8Ac2*	III	–	1.05	–
Neu5,9Ac2	I–IV	0.63	1.13	1.28
Neu5,7,9Ac3	I	0.70	β 1.14 α 1.07	1.53
Neu5,8,9Ac3*	III–IV	0.75	1.19	1.64
Neu5,7,8,9Ac4*	I–III	0.80	1.15	1.87
Neu5Gc	II–IV	0.35	β 1.81 α 1.90	1.43
Neu7Ac5Gc*	II	–	1.83	–
Neu9Ac5Gc	II–IV	0.55	2.04	1.75
Neu7,9Ac25Gc*	IV	0.70	2.01	2.06
Neu8,9Ac25Gc*	IV	–	1.99	–
Neu7,8,9Ac35Gc*	III	–	1.93	–

of the gas-chromatographic peaks of Neu5Ac, Neu5,7Ac$_2$, Neu5,7,9Ac$_3$, and Neu5Gc was based on this observation. The mass spectra of the α-anomers and β-anomers can mainly be distinguished by the ratio of the peak intensities of the fragments A (M-CH_3) and B (M-$COOCH_3$). In case of the α-anomers the intensity of fragment B increases relative to A (Fig. 4). Gas-chromatographic resolution of anomers was not observed for any of the trimethylsilyl ester, ether derivatives analyzed on OV-17.

Since the R_{Neu5Ac} values of a number of sialic acids do not differ very much (see Table 1), an identification based on GLC retention times alone is not sufficient. Especially for complex mixtures, analysis by GLC-MS is necessary. In this respect, the incorporation of capillary GLC-MS will be of high importance for the analysis of such complex mixtures [9].

Correlation of the TLC patterns with the identifications made by GLC-MS led to a set of R_F values for different sialic acids in the TLC system used (Table 1). The migration depends on the number and, in contradiction to data reported earlier [3], also on the position of O-acetyl groups. This is possibly due to the higher resolving power of the cellulose plates used in the present study. In general, N-glycoloyl derivatives show lower R_F values than the corresponding N-acetylated sialic acids. The necessity of two-dimensional TLC becomes evident in the case of Neu5,7Ac$_2$ and Neu9Ac5Gc, which have similar R_F values in the solvent system used. Discrimination can be achieved after two TLC runs under identical conditions with intermediate hydrolysis of O-acetyl groups by treatment with NH_3, yielding Neu5Ac and Neu5Gc, respectively, as parent sialic acids which can easily be distinguished on TLC [5].

Concluding Remarks

As has been demonstrated, the use of a flat gradient for the purification and prefractionation of sialic acids by anion-exchange column chromatography leads to a better separation of these compounds, which was not possible in the former fractionation procedure, where a gradient of 0–2 M formic acid was used [3]. By the modification described in this paper compounds which occur only in minor amounts in bovine submandibular gland glycoprotein can be detected in the presence of the predominant sialic acid species. These improved preparative and analytical techniques could also successfully be applied for the identification of sialic acids from other tissues (unpublished results).

The detection of 14 different sialic acids in bovine submandibular gland raises the question about the enzymic origin and biological role of this high variety. Studies related to these problems are in progress.

Thanks are due to Mr G. J. Gerwig, Miss C. Szeiki, and Mrs J. Riedemann for their excellent technical assistance. The financial support from the *Deutsche Forschungsgemeinschaft* (grants Re 445/2 and Scha 202/7

Table 2. m/z *values of the characteristic fragment ions A–H of the methyl esters, trimethylsilyl ethers of sialic acids from bovine submandibular gland glycoprotein*
Most of the spectra of these compounds have recently been depicted in a review on sialic acids [10]; *, new sialic acid; –, relative intensity below 3 %

Compound	A	B	C	D	E	F	G	H
Neu5Ac	668	624	478	298	317	205	173	400
Neu5,7Ac$_2$	638	594	–	–	317	205	173	400
Neu5,8Ac$_2$*	638	594	478	298	317	–	173	–
Neu5,9Ac$_2$	638	594	478	298	317	175	173	400
Neu5,7,9Ac$_3$	608	564	–	–	317	175	173	400
Neu5,8,9Ac$_3$*	608	564	478	298	317	–	173	–
Neu5,7,8,9Ac$_4$*	578	534	–	–	317	–	173	–
Neu5Gc	756	712	566	386	317	205	261	488
Neu7Ac5Gc*	726	682	–	–	317	205	261	488
Neu9Ac5Gc	726	682	566	386	317	175	261	488
Neu7,9Ac$_2$5Gc*	696	652	–	–	317	175	261	488
Neu8,9Ac$_2$5Gc*	696	652	566	386	317	–	261	–
Neu7,8,9Ac$_3$5Gc*	666	622	–	–	317	–	261	–

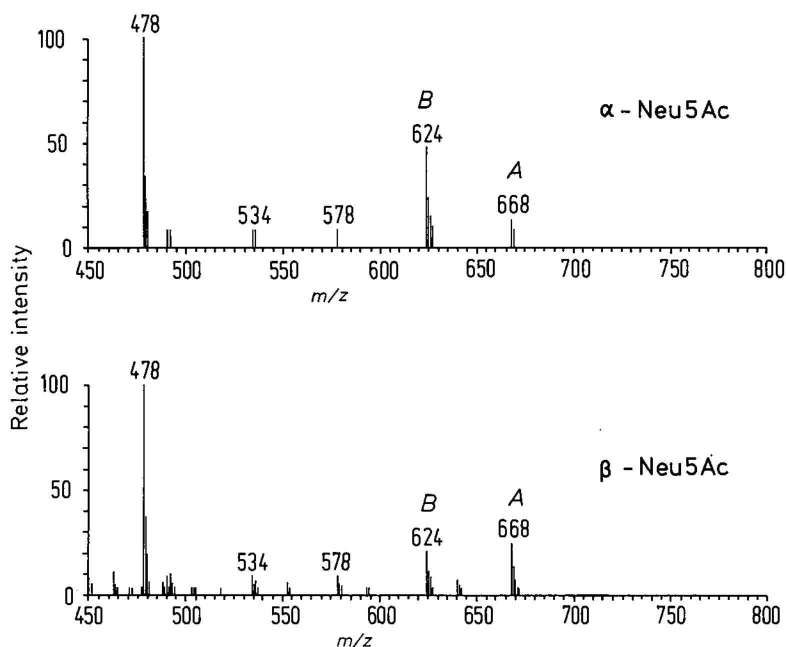


Fig. 4. *Partial mass spectra of the α and β anomer of Neu5Ac methyl ester, trimethylsilyl ether.* Fragments A ($M-CH_3$) and B ($M-COOH_3$) are indicated

and 9) and from the *Fonds der Chemischen Industrie* is greatly appreciated. This investigation was further supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

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