

The Sialic Acids of Bovine and Equine Submaxillary Mucins

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The sialic acids of bovine submaxillary mucin (BSM) were separated and isolated by the aid of paper column chromatography. The predominant sialic acids in BSM are an N,O-diacetylneuraminic acid (with the O-acetyl in C-7 position) and an N-acetyl-di-O-acetylneuraminic acid (with one of the O-acetyls at C-7 and the other at C-8 or C-9). Small quantities of N-glycolylneuraminic acid and N-acetylneuraminic acid were also found, the latter possibly formed from the diacetylneuraminic acid during the isolation procedure. In addition there appeared small amounts of a fifth sialic acid which contained O-acetyl and was possibly a labile isomer of the N-acetyl-7-O-acetylneuraminic acid.

The sialic acid of equine submaxillary mucin was isolated in pure crystalline form. It was identified as N-acetyl-4-O-acetylneuraminic acid.

In an earlier investigation¹ it was found that, on paper chromatography, several-times-recrystallized bovine submaxillary mucin (BSM) regularly yielded four spots as indicated by the orcinol or Ehrlich reagents. Numbering the fractions I—IV with increasing R_F -values, fraction II was the predominant one. The amounts of fraction II isolated at that time were too small to allow closer analysis. It could hardly be doubted, however, that this fraction was an O,N-diacetylneuraminic acid, the O-acetyl of which, according to the result of periodate oxidation, was at the C-7 position. The R_F of fraction I tallied with that of N-acetylneuraminic acid. The nature of the faint fraction III and of fraction IV remained unelucidated.

The heterogeneity of sialic acid preparations of BSM was also brought out by work of Klenk and Uhlenbruck². Applying a micro-method for the quantitative determination of the glycolyl group they obtained values corresponding to the presence of 21—25 % N-glycolylneuraminic acid in their preparations. We have arrived³ at somewhat lower values (13—17 %), the difference probably depending on somewhat differing methods of preparation.

The ratio between the different kinds of sialic acid may well be different in the mucin (BSM) from that in the several-times-recrystallized preparation of sialic acid obtained from it. The sialic acids differ in solubility, and the

O-acetyl groups are very easily split off³. The preparation method has therefore been modified in the endeavour to minimize the risk of losses in the mother liquors and by decomposition. In preparations obtained by the modified method fractions II and IV clearly predominate, and seem to be present in about equal amounts, perhaps with some preponderance of the latter. Both were isolated and analysed. It was confirmed that fraction II was an O,N-diacetylneuraminic acid. It appeared chromatographically uniform, but contained about 1—2 % glycollyl. Fraction IV proved to be an N-acetyl-di-O-acetylneuraminic acid. It was also found uniform on rechromatographing, and contained no glycollyl.

In the new preparations fraction I could be resolved chromatographically into two spots, one with the same R_F as N-glycollylneuraminic acid, the other moving as N-acetylneuraminic acid. Crystalline matter obtained from total fraction I gave the X-ray powder pattern of N-acetylneuraminic acid, indicating that this substance was the main component of the crystals (concerning mixed sialic acid crystals see Abrahamsson and Svennerholm⁶). Glycollyl determinations of total fraction I gave values corresponding to a content of 7—12 % of N-glycollylneuraminic acid. The N-glycollylneuraminic acid is probably, at least in the main, preformed, since fractions III and IV do not contain any glycollyl and fraction II contains little of it. The N-acetylneuraminic acid present may be preformed, or it may have been formed from the di- and triacetylneuraminic acids during the isolation procedure.

The di- and triacetylneuraminic acids are not stable in aqueous solutions at + 37°. When pure aqueous solutions of the diacetylneuraminic acid are kept for various lengths of time at + 37° and the ensuing changes followed with paper chromatography, there arises at first a new spot which moves with the R_F of fraction III. With longer times a spot with the R_F of N-acetylneuraminic acid appears which increases in size as the two others diminish. Attempts to obtain fraction III in crystalline form were unsuccessful. This fraction gives a positive ester reaction with the FeCl_3 -hydroxylamine test. It is probably a labile isomerization product of the diacetylneuraminic acid (formed by acetyl migration?). In similar experiments with the triacetylneuraminic acid the product which first formed had the R_F of the bovine diacetylneuraminic acid. Later spots appeared with the R_F -values of fraction III and N-acetylneuraminic acid, respectively. This result seems to indicate that one of the O-acetyl groups of the triacetylneuraminic acid has the same position as the O-acetyl of the bovine diacetylneuraminic acid, that is at C-7. Since the triacetylneuraminic acid is not oxidized by periodate, the second O-acetyl must be at C-8 or C-9.

In the earlier investigation¹ sialic acid from *equine* submaxillary mucin (ESM) was not obtained in a pure state. The analyses of the impure preparations suggested that this substance was an isomer to the bovine diacetylneuraminic acid. The purification procedure has now been improved and the nicely crystallizing substance obtained was chromatographically uniform.

Analysis of the substance has fully confirmed that it is an O,N-diacetylneuraminic acid. Contrary to the bovine diacetylneuraminic acid it consumes two molecules of periodate per molecule. Since the C-2 position is excluded, because this group is bound to hexosamine in the mucin, the result of the

periodate oxidation indicates that the equine sialic acid is N-acetyl-4-O-acetylneuraminic acid.

EXPERIMENTAL

1. Preparation of submaxillary mucins

The method is in principle the same as that used in earlier works from this laboratory¹. The procedure is now described in more detail, to facilitate its reproduction.

Fresh glands are freed from adherent lymph glands, fat, and connective tissue, and are then frozen. Batches of 3 kg of frozen glands are cut into cubes of about 2 cm³. The cut glands are then barely covered with distilled water, and kept at + 4° for 24 h, with occasional stirring. The cubes are removed by pouring the mixture through a stainless steel cone perforated with holes about 4 mm in diameter. The "filtrate" is freed from coarse particles by centrifuging. The first extract is usually strongly tinged by haemoglobin, and contains in addition much protein other than mucin, which may disturb the precipitation of the latter. It is therefore best discarded. The glands may be extracted at least 5–7 times in this way.

Dilute hydrochloric acid is added to the extract until the mucin just dissolves on the acid side of its isoelectric zone. The amount of HCl required may vary, and is conveniently determined in a preliminary test on a few ml of the extract. The mucin is precipitated by dilution with a few volumes of water. If the solution is vigorously stirred during and after the process of dilution, the mucin precipitate as a rule coalesces and adheres as an elastic clot to the spatula or rod used for stirring. The mucin is then dehydrated by alcohol, and torn into small pieces during the procedure. It is then ground to a powder, and freed from fatty impurities by extraction with ether.

2. Isolation of the BSM sialic acids

40 g of mucin is placed in a 2-litre flask. Small portions of water are added, and the whole vigorously shaken until a viscid suspension is obtained. The suspension is then made up to 400–500 ml water with gentle warming and constant shaking. The suspension, which has a pH of 3.3–3.4, is heated for 1 h in the boiling water bath. After cooling to room temperature the mixture is centrifuged, and the undissolved residue washed with 200 ml water. The supernatants are combined and freeze-dried.

The residue is repeatedly (6 times) extracted with methanol for 4–5 h at + 4°. Since the tri- and di-acetylneuraminic acids dissolve much more readily in methanol than do the N-acetyl- and N-glycollyneuraminic acids, the first extracts of the residue contain the main part of the two former kinds of sialic acid and nothing or very little of the two latter, which predominate in the last extracts (Fig. 1). In order to minimize methyl ester formation each extract is evaporated below 0° in the freeze-drying apparatus. The residues are dissolved in small quantities of methanol, and the solutions then combined and brought to dryness below 0°. The final residue thus obtained is dissolved in a minimum of water (about 2 ml); 30 ml of methanol is added, followed by 90 ml of ether. An amorphous precipitate formed is immediately filtered off. The filtrate is evaporated in the freeze-drying apparatus. The residue is dissolved in 5 ml of water-*n*-butanol-acetic acid (5:4:1), and then chromatographed on a paper roll (75 × 6 cm) in the same solvent. The LKB Chromax pressurized paper column system* was used. The eluate is collected in fractions of about 20 ml. Of each or each second fraction 0.5 ml is taken for sialic acid analysis (with the Ehrlich reagent). Fractions II, III, and IV are not completely separated in this procedure, but the separation is sufficient for the preparation of fractions II and IV in pure state.

The fractions containing the di- and triacetylneuraminic acid are pooled separately, and evaporated to dryness in the freeze-dryer. In order to remove the last traces of butanol the residues are dissolved in about 100 ml of methanol, and the solution evaporated to dryness in the same manner.

* May be obtained from AB LKB-Produkter, Stockholm, Sweden.

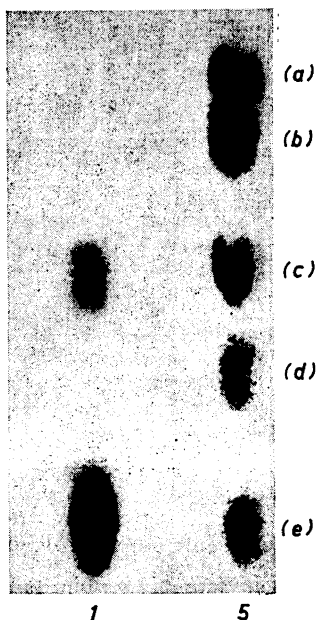


Fig. 1. Paper chromatogram of the first (1) and fifth (5) methanol extract of sialic acids of BSM. Solvent: *n*-butanol-pyridine-water, 6:4:3.

(a) N-glycolylneuraminic acid, (b) N-acetylneuraminic acid, (c) diacetylneuraminic acid, (d) "fraction III", (e) triacetylneuraminic acid.

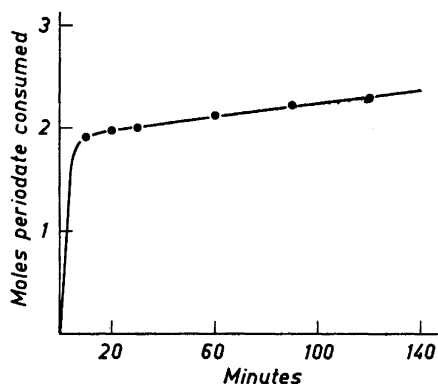


Fig. 2. Periodate oxidation of the sialic acid of equine submaxillary mucin in acetate buffer of pH 4.4 at +8–10°. Moles periodate consumed per mole of substance oxidized.

For crystallization fraction II is dissolved in 1–2 ml water, fraction IV in at most 1 ml water; 30 ml of methanol is added, followed by 90 ml of ether. Light petroleum is then added drop by drop to the filtrate until faint turbidity appears. Crystallization takes place at room temperature or in the refrigerator. More substance crystallizes on each addition of a few drops of light petroleum during the next few days. The formation of crystals may continue for a week or more. (If too much light petroleum is added at one time the sialic acid comes out as an oil.) Recrystallization is performed in the same way. The yield of the once-recrystallized substances is 150–200 mg of diacetylneuraminic acid and about 500 mg of triacetylneuraminic acid.

3. Analysis of the BSM sialic acids

The *diacetylneuraminic acid* crystallizes with 1 molecule crystal water in rectangular plates, just as the crude substance obtained in the earlier investigation¹. The substance decomposed with browning at 138–140°. $[\alpha]_D^{25}(\text{H}_2\text{O}) + 6 \pm 2^\circ$. These values differ slightly from those originally given for this substance¹.

The nitrogen determinations were carried out by Dumas' method, the total acetyl as described by Friedrich and Rapaport⁴, and the O-acetyl by Hestrin's method⁵. Two

Nitrogen analyses,		
$C_{13}H_{21}NO_{10}$ (351)	Calc.	N 3.99
(dried at 100° <i>in vacuo</i>)	Found 1.	3.98
		3.98
	2.	3.84
		3.82
$C_{13}H_{21}NO_{10} \cdot H_2O$ (369)	Calc.	N 3.79
(dried in desiccator)	Found	3.74
		3.77
Total acetyl,	Calc.	23.3
$C_{13}H_{21}NO_{10} \cdot H_2O$	Found	21.2
		21.4
		22.0
O-acetyl,	Calc.	11.6
$C_{13}H_{21}NO_{10} \cdot H_2O$	Found	10.7

glycolyl determinations (method of Klenk and Uhlenbruck²) gave values between 1.3 and 2.2 %. The X-ray powder pattern was identical with that of the crude product earlier investigated¹.

The *triacetylneuraminic acid* crystallized in needles or prisms with one molecule of crystal methanol, which did not disappear on heating at + 70° for several hours. It was driven off by heating *in vacuo* at 100° for 10 h. Methoxyl determination on the substance dried at 70° gave 7.4 % (calc. for $C_{15}H_{23}NO_{11} \cdot CH_3OH$ 7.4 %), whereas the substance dried at 100° gave no methoxyl value. It does not consume any periodate at pH 4.4 and + 10°.

The substance is more easily soluble in methanol than the other sialic acids. It melts with gas evolution but without discoloration at 130–131°. $[\alpha]_D^{25}$ (H_2O) + 9 ± 2°. The elementary and acetyl analyses agreed reasonably with the formula $C_{15}H_{23}NO_{11} \cdot CH_3OH$.

$C_{15}H_{23}NO_{11} \cdot CH_3OH$ (425)	Calc.	C 45.17	H 6.40	N 3.29
	Found 1.	44.83	6.53	—
		44.85	6.50	—
	2.	44.60	6.45	3.41
		44.58	6.43	3.42
	3.	44.77	6.43	3.30
		44.66	6.46	3.31

Total acetyl,	Calc. (three acetyls)	30.35
	Found	29.8
		32.9
O-acetyl,	Calc. (two acetyls)	20.21
	Found	20.6
		20.9

3. Isolation of the *equine diacetylneuraminic acid*

40 g of mucin is suspended in 400–500 ml water as described above for BSM. The pH is then adjusted to 2.5, and the suspension heated for 1 h in the boiling water bath with occasional shaking. The pH tends to rise, and should therefore be adjusted to pH 2.5 two or three times during the heating. After cooling to room temperature the mixture is centrifuged, and the undissolved residue washed with 200 ml water. The supernatants are combined and neutralized to pH 3.5 with dilute barium hydroxide. The new mixture is then freeze-dried without previous filtration.

The residue is repeatedly (5–6 times) extracted with methanol for 4–5 h at + 4°. In order to minimize methyl ester formation each extract is evaporated below 0° in the freeze-dryer.

The residues are dissolved in small quantities of methanol. The solutions are combined and brought to dryness in the freeze-dryer. The final residue thus obtained is dissolved in a minimum of water (4–5 ml), 30 ml methanol is added, followed by ether and light petroleum in the same manner as described for the BSM sialic acid. On recrystallization the addition of light petroleum is superfluous. The pure substance may be recrystallized from methanol without addition of ether. They equine sialic acid crystallizes in long needles. 40 g mucin yields 0.4–0.5 g of recrystallized substance.

4. Analyses of the equine diacetylneuraminic acid

The substance decomposes with browning at about + 200°. $[\alpha]_D^{22}$ (H₂O) $-62 \pm 1^\circ$. These values are slightly higher than those reported for the impure substance ¹.

C ₁₃ H ₂₁ NO ₁₀ (351),	Calc.	C 44.44	H 6.03	N 3.99
	Found	44.34	6.26	4.04
		44.18	6.17	4.07
Total acetyl,	Calc. (two acetyls)	24.5		
	Found	21.0		
O-acetyl,	Calc. (one acetyl)	21.5		
	Found	11.6		

The substance contained no appreciable amounts of methoxyl or glycolyl groups. The X-ray powder diagram was identical with that of the impure product given in the earlier report ¹. Periodate oxidation was carried out at pH 4.4 and + 8–10°, as earlier ¹ described. The result of one such experiment is given in Fig. 2: 2 moles of periodate are evidently consumed per mole sialic acid.

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