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5-Bromo-indol-3-yl 5-Acetamido-3,5-dideoxy-α-D-glycero-D-galactononulopyranosidonic Acid, a Novel Chromogenic Substrate for the Staining of Sialidase Activity

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The purification and characterisation of viral, bacterial and mammalian sialidases (EC 3.2.1.18, neuraminidases, neuraminosylglycohydrolases) prompted a search for a colorimetric technique to localize the enzymes on electropherograms. The 5-bromo substituted indol-3-yl α-ketoside of 5-*N*-acetyl-D-neuraminic acid (BIN), the synthesis of which is described here, seemed to be the appropriate substrate, because of its relative ease of enzymatic hydrolysis to 5-*N*-acetyl-D-neuraminic acid and 5-bromo-indoxyl. The latter is readily transformed to the insoluble, intensely coloured 5,5′-dibromo-indigo. This precipitates and can be seen readily at the sites of enzymatic activity. The new substrate is of definitive advantage as it provides a simple and direct method for the demonstration of sialidases without the need of a coupling reaction.

Sialidases (EC 3.2.1.18), which catalyze the hydrolysis of sialic acid α-ketosides, have been detected in viruses [1], bacteria [2] and mammalian tissues [3]. The important biological role played by these enzymes, both in the normal state and in pathology, has been established. Biochemical investigations with various synthetic and naturally occurring α-ketosides of 5-*N*-acetyl-D-neuraminic acid (Neu5Ac) have shown that sialidases exist in different cell compartments. Following centrifugation of tissue homogenates the enzymes were found in the lysosomal, microsomal, mitochondrial and synaptosomal fractions as well as in the supernatant with different properties [4].

Abbreviations: Neu5Ac, 5-*N*-acetyl-D-neuraminic acid; BIN, 5-bromo-indol-3-yl α-ketoside of *N*-acetyl-D-neuraminic acid.

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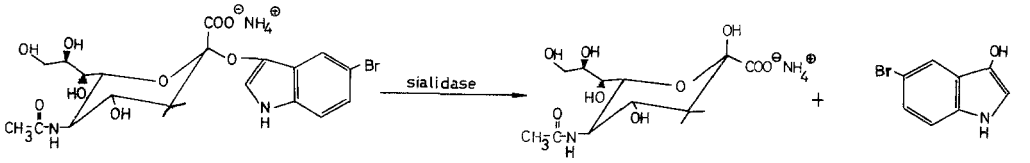
1. primary reaction (enzyme reaction):



2. secondary reaction (precipitation) :

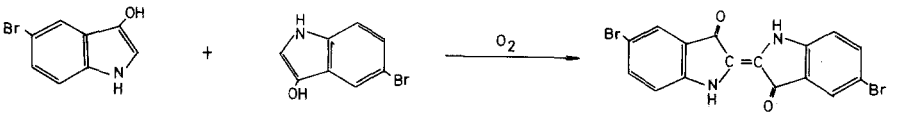


Figure 1. Indigogenic staining.

Studies on the purification and characterisation of sialidases of different origins prompted a search for chromogenic substrates for the localization of sialidase activity on electropherograms. Current methods employ mainly a 3-methoxyphenyl α-ketoside of 5-*N*-acetyl-D-neuraminic acid in conjunction with an azo- [5] or 4-amino-antipyrine [6] coupling procedure.

Our previous reports described the application of the indigogenic principle to the histochemical demonstration of sialidases in mammalian tissues [7, 8].

The present study extends the same chromogenic reaction sequence that underlies this principle to the precise localisation of sialidase activity in electropherograms. The chromogenic reaction sequence underlying what has come to be known as "indigogenic staining" (Fig. 1) is initiated by enzymatic release of an intermediate 5-bromo-indoxyl. This intermediate is rapidly and irreversibly transformed by air oxidation to an insoluble and highly coloured 5,5'-dibromo-indigo, which is deposited at the sites of the enzyme activity. The same underlying principle applies for the use of 5-bromo-4-chloro indol-3-yl phosphate and 5-bromo-4-chloro indol-3-yl β-galactoside in histochemistry.

The synthesis of the new substrate 5-bromo-indol-3-yl 5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-nonulopyranosidonic acid (BIN), and its use to distinguish sialidases of different origin is the subject of this communication.

Materials and Methods

*Methyl (5-Bromo-indol-3-yl 5-Acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-α-D-glycero-D-galactononulopyranosidonate (**1**)*

A solution of sodium methoxide (2.16 g) and 5-bromo-indoxyl 1,3-diacetate [9] (8 g) in *N,N'*-dimethylformamide (25 ml) was gassed for 30 min with a stream of dry nitrogen and then stirred magnetically at room temperature. After 2 h the reaction mixture was poured on to freshly prepared methyl(5-acetamido-4,7,8,9-tetra-*O*-acetylchloro-2,3,5-trideoxy-β-D-glycero-D-galactononulopyranosidonate [10] (1.9 g) and stirred magnetically at room temperature for 12 h. TLC then showed that the glycosyl chloride had been consumed. The blue-green solution was evaporated to dryness under diminished pressure (traces of *N,N'*-dimethylformamide being removed by repeated evaporations of xylene from the product), and the residue was dissolved in ethyl acetate. After filtration, the solution was concentrated to a small volume, and chromatographed on a column of silica gel 60 (Merck, Darmstadt, W. Germany). The fractions were examined by TLC and those containing only the chromogenic substance with R_{Neu5Ac} = 1.53 (propanol/water, 7/3 by vol) were combined and concentrated to a volume of 100 ml.

This solution was shaken with water (3 × 100 ml). The organic layer was dried with magnesium sulfate and concentrated to dryness. After dissolution in a small volume of acetone the residue (435 mg, 16%) crystallized on treatment with diethyl ether/petroleum ether (40°) to give a pale yellow solid. Physical constants and analysis are given in Table 1.

In a similar run we were also able to isolate the *N*(1)-acetyl derivative of **1** as a colourless glass ($[\alpha]_D$ + 13.8 (22°C), c 1.4, methanol), ^{1}H-NMR data (C^2HCl_3) δ 8.33 (d, 1H, indolyl H-7), 7.68 (d, 1H, indolyl H-4), 7.44 (d, 1H, indolyl H-6), 7.25 (s, 1H, indolyl H-2), 5.44 (ddd, 1H, H-8), 5.31 (dd, 1H, H-7), 5.22 (d, 1H, NH), 4.98 (ddd, 1H, H-4), 4.32 (dd, 1H, H-6), 4.28 (dd, 1H, H-9), 4.11 (ddd, 1H, H-5), 4.08 (dd, 1H, H-9'), 3.62 (s, 3H, $COOCH_3$), 2.86 (dd, 1H, *J*3a/3e 12.8, *J*3e/4 4.9 Hz, H-3e), 2.67 (s, 3H, indolyl *N*-Ac), 2.31 (dd, 1H, *J*3a/3e 12.8, *J*3a/4 12.6 Hz, H-3a), 2.16, 2.12, 2.07, 2.02 and 1.93 (5s, 15H, 5Ac).

*5-Bromo-indol-3-yl 5-Acetamido-3,5-dideoxy-α-D-glycero-D-galactononulopyranosidonic acid (BIN) (**2**)*

The methylester glycoside **1** (49 mg) was dissolved in methanol (20 ml), treated dropwise with 1 M sodium hydroxide (5 ml), and the solution was stored at room temperature for 30 min. After cooling to 0°C, sodium ions were removed by addition of Dowex 50 W-X8 (H^+) resin to pH 4 followed by filtration. The combined filtrates were evaporated to dryness. Freeze-drying of the residue gave a chromatographically and electrophoretically homogeneous product as a colourless powder (30 mg, 80%). Physical constants and analysis are given in Table 1.

Sialidases

Culture filtrate (lyophilized ultrafiltrate) of *Vibrio cholerae* was kindly supplied by Behringwerke AG (Marburg, W. Germany). Purified *Vibrio cholerae* neuraminidase (1 ml containing 1 U, manufacturer's specification) was purchased from Behringwerke AG. *Clostridium perfringens* sialidase (1 U/mg) was obtained from Boehringer Mannheim

Table 1. 5-Bromo-indol-3-yl α-*N*-acetyl-4, 7, 8, 9-tetra-*O*-acetyl-D-neuraminate (**1**) and 5-*N*-acetyl-D-neuraminic acid (2)

		Peracetylated methylester **1**	Unprotected compund **2**
Formula (MW)		$C_{28}H_{33}N_2O_{13}Br$ (676.5)	$C_{18}H_{23}N_2O_9Br.H_2O$ (514.3)
Calculated (%)	C	49.70	42.60
	H	4.91	4.93
	N	4.12	5.51
Found (%)	C	49.04	42.33
	H	4.88	4.91
	N	3.55	5.29
Melting point (°C)		165-167	luophilized
Optical rotation $[\alpha]D_{25}$		+13.3	+12.0
g/l		2.8	2.1
solvent		methanol	methanol

(Mannheim, W. Germany). *Streptococcus (Diplococcus) pneumoniae* sialidase was kindly provided by Dr. H. Pech (Hygiene-Institut, Universität Heidelberg).

Electrophoresis

Polyacrylamide gel electrophoresis was carried out under standard conditions at 4°C for 1-2 h at 5 mA per tube. Polyacrylamide gels (7.5% and 3.75%) were used at pH 7.5 [11] and 4.3 [12]. In addition, electrophoresis was performed on 7.5% polyacrylamide gel at pH 8.5 [13]. *Vibrio cholerae* or *Streptococcus pneumoniae* culture filtrate preparation (5-20 μl, 1-2 mU, 15-60 μg protein) or 25 μl of the commercial preparation of *Vibrio cholerae* and *Clostridium perfringens* were applied to the gels in an aqueous solution containing 30% (w/v) sucrose.

Isoelectrofocusing was performed according to the literature [14]. Gradients of pH 2-11 and pH 4-6 were established by a concentration of 1% Ampholine (LKB, Bromma, Sweden). Gels were stained with either Amido black 10B (Serva, Heidelberg, W. Germany) or Coomassie blue (Sigma, München, W. Germany).

For the cellulose acetate electrophoresis of commercial *Vibrio cholerae* neuraminidase, 0.036 M sodium barbital buffer pH 8.5 was employed. The commercial solution (20 μl) was applied to a 3.9 cm wide strip of cellulose acetate. Bromphenol blue (Merck) was used as a marker for the buffer front moving towards the anode and run at room temperature for 1-5 h at 5 V/cm.

Specific Staining of the Sialidases

After disc electrophoresis the bands of sialidase activity were detected by incubating the gels for 3-4 h at 37°C in (a) 0.05 M sodium acetate buffer (pH 5.5) containing NaCl (9 mg/ml), $CaCl_2$ (1 mg/ml) and 5-bromo-indol-3-yl 5-acetamido-3,5-dideoxy-α-D-glycero-D-galactononulopyranosidonic acid (1.5 mg/ml) or (b) 0.5 M sodium phosphate buffer (pH 6.0) and BIN (1.5 mg/ml).

In order to detect sialidase activity in cellulose acetate strips they were incubated for 15-20 minutes at 37°C with a filter paper pressed against them which was soaked with 1.5 mg/ml solutions of BIN in 0.1 M sodium phosphate buffer pH 6.4. After air-drying the strips for at least 1 h, dark blue bands developed at the sites of sialidase activity.

Results and Discussion

The synthesis of **1**, which involves the reaction of the glycosyl chloride of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-β-D-glycero-D-galactononulopyranosonate with 5-bromo-indoxyl-1,3-diacetate in the presence of sodium methoxide as the key coupling-step, afforded the 5-bromo-indolyl α-ketoside **1** in 16% yield.

The high positive rotation ($[\alpha]_D$ + 13.3 at 25°C) of **1** suggests the α-configuration and this is supported by one piece of NMR evidence. The chemical shift for H-3e at δ 2.86 is diagnostic of the α-configuration [15-17]. In additio, the ketoside **2** was found to be completely cleaved by *Vibrio cholerae* sialidase, which is known to split α-glycosides.

The 5-bromo substituted indol-3-yl α-ketoside of 5-*N*-acetyl-D-neuraminic acid proved to be an appropriate substrate for the tracing of sialidase activity, because of its relative ease of enzymatic hydrolysis as well as the nature of the final reaction product which is highly coloured, particulate and insoluble.

The principle of the staining reaction is similar to that of the 5-bromo-indol-3-yl-acetate [9]. Sialidases cause hydrolysis of the colourless 5-bromo-indol-3-yl 5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-nonulopyranosidonic acid (BIN). The unstable intermediate 5-bromo-indoxyl is readily transformed to the insoluble blue-green 5,5'-dibromo-indigo. This precipitates and can be seen readily at sites of enzymatic activity in the samples.

The results with BIN and three different sialidases are shown diagramatically in Fig. 2 and 3. No attempt was made in the present study to obtain a quantitative estimate of the amount of enzyme present in a band.

The advantage of BIN as a chromogenic substrate for sialidases resides in the fact that 5-bromo-indoxyl, enzymatically released, readily forms 5,5'-dibromo-indigo even at acid pH. BIN requires neither a diazo coupler nor pH adjustment to bring out the colour of the final reaction product. Since other proteins are not stained by BIN, they can be readily distinguished from the specific action of sialidases.

The commercial samples of *Vibrio cholerae* and *Clostridium perfringens* sialidases invariably produced a single dark blue band when incubated with BIN after disc electrophoresis in 7.5% polyacrylamide at either pH 8.5 or pH 4.3 or in 3.75% polyacrylamide

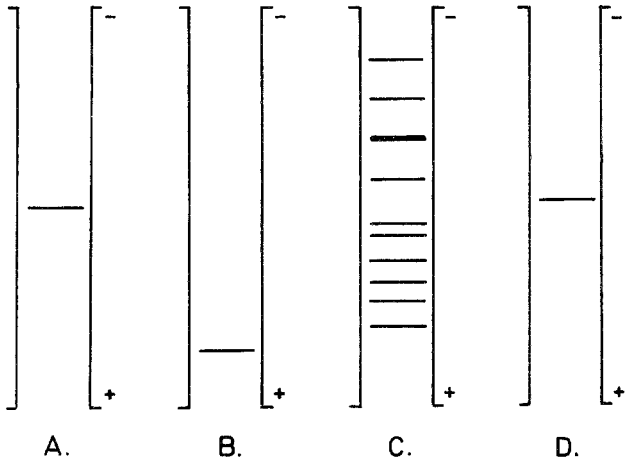


Figure 2. Disc electrophoresis of *Vibrio cholerae* and *Clostridium perfringens* sialidases in 7.5% polyacrylamide ("running pH" 8.5).
Gel A: Commercial *V. cholerae* sialidase, staining with BIN*.
Gel B: Commercial *Clostridium perfringens* sialidase staining with BIN*.
Gel C + D: Crude *V. cholerae* sialidase, staining with BIN (C) and amidoblack (D).
*Staining of protein was not possible, because of the low protein content.

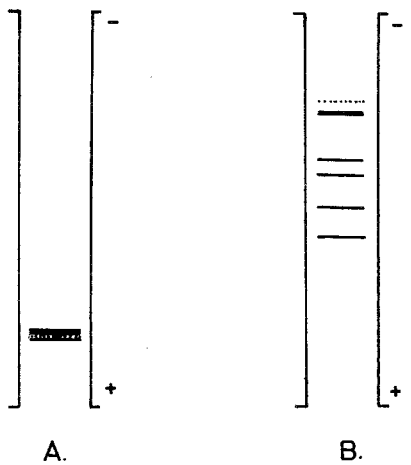


Figure 3. Crude sialidases from *Streptococcus pneumoniae* Type 1 (strain 202/78). A: disc electrophoresis ("running pH" 8.5). B: isoelectric focusing (gradient pH 2-11).

at pH 4.3. However, the protein content of these two enzyme preparations was too low to be detected by amido black or Coomassie blue, repectively (Fig. 2A + 2B).

In contrast, a crude preparation of *Vibrio cholerae* sialidase contained a large amount of contaminating proteins which offer, after disc electrophoresis and subsequent indication with amido black, one major and several minor bands. When developing the sialidase activity with BIN as substrate, there appeared a single additional band due to enzyme action after electrophoresis at pH 8.5 in 7.5% polyacrylamide (Fig. 2C and D). A 3.75% polyacrylamide gel, on the other hand, did not allow the enzymes to enter the gel. The sialidase band was found on the very top of the tube, thus indicating that the enzyme had failed to penetrate into the small pore gel.

The tracing of sialidase activity on cellulose acetate in the presence of BIN has so far failed in our hands. Under the conditions used, the enzyme activity formed a rather broad band, indicating that despite a rather short incubation time the bacterial sialidase had spread by diffusion.

Streptococcus (Diplococcus) pneumoniae is known to be a source of sialidases; in one case the existence of isoenzymes was reported [13]. In the present studies we have observed multiple forms in the culture filtrate of *Streptococcus pneumoniae* type 1 (strain 202/78). Disc electrophoresis ("running pH" 8.5) of the crude preparation showed the presence of three enzymes, while in the isoelectric focusing experiments (gradient pH 2-11) at least six bands of enzymatic activity have been found to appear (Fig. 3). A narrower pH gradient could be used if required, and would be expected to give enhanced resolution.

The results so far obtained concerning the electrophoretic identification and separation of sialidases using the chromogenic substrate, BIN, as an analytical tool, strongly suggest that this method may prove valuable in further investigations on mammalian, bacterial and viral sialidases isoenzymes. This new substrate is a definite advance in tracing sialidase activity, beacause of the fine and particulate reaction product that it affords, giving readily detectable blue-green deposition and as it provides a simple and direct method for demonstration of this enzyme without the need for a coupling reaction.

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