Neuraminidase Inhibition by Chemically Sulphated Glycopeptides

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Chemically sulphated glycopeptides (derived from pig duodenal mucosa) inhibited *Clostridium perfringens* neuraminidase (EC 3.2.1.18) activity in a pH-dependent manner. Analysis of inhibition kinetics data indicated that, although the enzyme inhibition could not be categorized into any of the classical types of inhibition, it could be interpreted as a function of the size and shape of the substrates used. The enzyme activity was inhibited by 86% and 40% when tested with bovine submaxillary-gland mucin (mol.wt. 4×10^5 – 40×10^5) and *N*-acetylneuraminyl-lactose (mol.wt. 633) as substrates respectively. Presence of sulphated glycopeptide did not affect the binding of *N*-acetylneuraminic acid (mol.wt. 309), a competitive inhibitor of *Vibrio cholerae* neuraminidase, to the enzyme active site. The enzyme inhibition was thus considered to be due to steric hindrance as a consequence of the non-specific interactions between the enzyme molecule and polyanionic sulphated glycopeptides affecting the differential accessibility of the substrate molecules to the enzyme active site. The enzyme-inhibitor interaction could be suppressed by rapid and many-fold dilution of the reaction mixture, by concurrent addition of the inactive enzyme or by partial removal of the sulphate esters from the sulphated glycopeptide molecule by the action of *Helix pomatia* arylsulphatase (EC 3.1.6.1).

It had been observed that sialoglycopeptides prepared without removal of glycosaminoglycans showed little reaction with calf brain neuraminidase and that chondroitin sulphate and to a greater extent heparin inhibited the enzyme action when gangliosides or sialoglycopeptides were used as substrates (Heijlman, 1974). Similar observations that the presence of sulphated glycosaminoglycans induced a strong pH-dependent inhibition of some 30 lysosomal enzymes including neuraminidase has also been reported (Avila & Convit, 1975, 1976). Neuraminidase inhibition by these sulphated glycosaminoglycans and by some other polyanionic macromolecules such as dextran sulphate, RNA and DNA (Drzeniek, 1966) have been considered, as a result of interaction between anionic groups of these molecules and the cationic groups on the enzyme.

The recent interest in the effect of some sulphated glycopeptides on neuraminidase activity was evoked by the findings that the chemical sulphation of a sialoglycopeptide separated from pig duodenal mucosa improved its anti-ulcerogenic and anti-inflammatory action (Bertellini *et al.*, 1971*a,b*) and also rendered it with pepsin-inhibition activity (Lietti *et al.*, 1971; Kent *et al.*, 1978). Our preliminary findings showed that this chemically sulphated glycopeptide acted as an inhibitor of neuraminidase with respect to the unsulphated sialoglycopeptide when used as a substrate (Kent *et al.*, 1978). It was thus considered important to further investigate the role of substitu-

tion of *O*-sulphate esters in sialoglycopeptides with a view to their behaviour towards neuraminidase. In the present paper we report a comparative study on the effect of sulphated glycopeptides on the reaction kinetics of neuraminidase (EC 3.2.1.18) with *N*-acetylneuraminyl-lactose and bovine submaxillary-gland mucin as substrates.

Materials and Methods

Clostridium perfringens and *Vibrio cholerae* neuraminidases were purchased from Boehringer Corporation, London W.5, U.K., and BDH Chemicals, Poole, Dorset, U.K., and were diluted in the appropriate buffers as required. *Helix pomatia* arylsulphatase (EC 3.1.6.1) was obtained from Sigma. *N*-Acetylneuraminyl-lactose, mol.wt. 633 [*N*-acetylneuraminyl-(2 → 3)- and (2 → 6)- β -galactopyranosyl-(1 → 4)-D-glucopyranose; type I, from bovine colostrum; Sigma] was used as purchased without further purification or fractionation. This compound is a mixture of approximately 85% of (2 → 3)-isomer and 15% of (2 → 6)-isomer according to the manufacturer's analysis [see the following paper (Mian *et al.*, 1979) for further details]. Bovine submaxillary-gland mucin (type I), also obtained from Sigma, was reported to be of mol.wt. 4×10^5 – 40×10^5 (Downs & Pigman, 1969).

All samples of sialoglycopeptides and sulphated glycopeptides, supplied by Crinos Biological Research Laboratories, Como, Italy, were dissolved in

water and dialysed against several changes of water before freeze-drying for their use in the present work.

Determination of neuraminidase activity

Assays of the neuraminidase activity towards a variety of substrates were carried out in 0.05M-sodium acetate/acetic acid buffer, pH 5.0 (Gomori, 1955) at 37°C for 1 h. The final concentration of the enzyme protein in 0.2 ml of reaction mixture was 1–2 µg. The incubations were terminated by either immersing the tightly sealed tubes in a boiling water bath for 2 min or the addition of 0.2 ml of phosphotungstic acid reagent (5 g/100 ml; 2.5M-HCl). The assay tubes were then centrifuged at 500g for 10 min and portions of the supernatant were taken either for direct estimation of the released *N*-acetylneuraminic acid or its separation from the remainder of the reaction mixture. *N*-Acetylneuraminic acid released by acid hydrolysis of the samples or by neuraminidase reaction was separated by ion-exchange chromatography on Dowex 1 (X8; formate form) as described by Cook (1976).

Determination of N-acetylneuraminic acid

Estimation of free *N*-acetylneuraminic acid was carried out by the 2-thiobarbituric acid method of Warren (1959) and of total free and combined *N*-acetylneuraminic acid by the resorcinol method of Svennerholm (1957).

Estimation of sulphate

Sulphate contents of the samples were determined by the method of Mende & Whitney (1978) following their hydrolysis in 25% formic acid (v/v) for 24 h at 100°C.

Carbohydrate analysis

The carbohydrate composition of the dialysed freeze-dried samples was analysed after methanolysis by the method of Clamp *et al.* (1971) by using g.l.c.

Amino acid analysis

Amino acid analysis was carried out on a Locarte automatic amino acid analyser after hydrolysis in 6M-HCl for 24 h at 105°C in evacuated sealed ampules.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as standard.

Gel filtration

Gel filtration of the dialysed samples of glycopeptides was carried out on Bio-Gel A-15m (200–400 mesh) obtained from Bio-Rad Laboratories, St. Albans, Herts., U.K. Columns (1.25 cm × 86 cm) were equilibrated with the eluting buffers. The buffers used were (a) 50 mM-Tris/acetate, pH 8.0, containing

100 mM-NaCl and 0.02% NaN₃ or (b) 50 mM-Tris/HCl, pH 8.0, containing either 4 M-guanidine hydrochloride or 8 M-urea, depending on the nature of the experiments. The columns were eluted at a flow rate of 2.5 ml/h and the fractions were tested for carbohydrate-positive material.

Cellulose acetate electrophoresis

Samples were characterized by zone-electrophoresis on cellulose acetate strips in 70 mM-sodium barbitone/barbituric acid buffer, pH 8.6, for 45 min at 22 V/cm. The strips were stained with 1% Alcian Blue in 2.5% acetic acid or with periodic acid/Schiff reagent.

Kinetics of neuraminidase reaction

Kinetic experiments were performed by using various concentrations of substrates or inhibitors under other variable conditions described below for individual experiments. The K_m and V_{max} values were calculated by Lineweaver–Burk plots as described by Dixon & Webb (1964a).

Results and Discussion

Characterization of sialoglycopeptides and sulphated glycopeptides

Fractionation of the dialysed samples on Bio-Gel agarose A-15m showed that both sialoglycopeptides

Fig. 1. Gel filtration of sialoglycopeptide and sulphated glycopeptide on Bio-Gel A-15m

Gel filtration of exhaustively dialysed samples of glycopeptides was carried out on Bio-Gel A-15m (200–400 mesh) columns (86 cm × 1.25 cm) equilibrated with 50 mM-Tris/acetate, pH 8.0, containing 100 mM-NaCl and 0.02% NaN₃. The columns were loaded with 5 mg of (a) sialoglycopeptide (GLP-400) and (b) sulphated glycopeptide (GLPS-341) and were eluted with the above buffer at a flow rate of 2.5 ml/h. The fractions (3.0 ml each) eluted were tested for carbohydrates by using anthrone reagent. The elution profiles were expressed as arbitrary units of anthrone reagent test.

Table 1. Carbohydrate and amino acid analysis of sialoglycoproteins and sulphated glycopeptides

	Carbohydrate or amino acid* content ($\mu\text{mol/g dry wt.}$)					
	Sialoglycopeptides			Sulphated glycopeptides		
	GLP-400	GV-023	GB.045	GLPS-341	GLPS-283	GO.075
Fucose	246	—	—	190	140	254
Galactose	703	10	90	528	730	—
Glucose	—	96	—	—	—	—
Mannose	—	10	100	—	—	—
N-Acetylgalactosamine	607	233	125	490	391	720
N-Acetylglucosamine	695	—	675	446	341	666
Aspartic acid	56	42	28	55	45	54
Threonine	568	420	252	538	478	530
Serine	308	234	127	300	275	300
Glutamic acid	86	68	38	89	68	80
Proline	362	265	136	341	238	320
Glycine	66	50	23	68	51	60
Alanine	150	106	53	135	112	125
Valine	93	68	34	90	76	90
Isoleucine	18	12	7	16	14	15
Leucine	64	45	24	58	46	50
Histidine	66	41	22	59	43	54
Arginine	38	25	11	36	28	30
Lysine	18	10	7	15	12	15

* Tyrosine and phenylalanine have not so far been estimated owing to their proximity to the hexosamines in the normal elution profile. No cysteine or methionine residues were detected (in their oxidized form) after performic acid oxidation.

Fig. 2. Double-reciprocal plot of *C. perfringens* neuraminidase activity against *N*-acetylneuraminyl-lactose concentration

Neuraminidase activity was assayed as described in the text. Velocities are expressed as μmol of *N*-acetylneuraminic acid released/h per mg of enzyme protein.

conditions. The electrophoretic examination of these samples also demonstrated that these macromolecules migrated as single components on cellulose acetate strips and were periodic acid/Schiff-positive. Treatment with 4M-guanidine hydrochloride or 8M-urea did not cause any dissociation of these macromolecules, as indicated by subsequent gel-filtration chromatography in the presence of these reagents. In a structural examination of a sialoglycopeptide (GLP-400), the carbohydrate side chains were cleaved from the peptide by the action of sodium borohydride in alkaline solution with concomitant destruction of serine and threonine residues and quantitative formation of *N*-acetylgalactosaminol. The oligosaccharide chains, separable by electrophoresis, were of at least three types, terminating in sialic acid, fucose and galactose respectively, and attached to the peptide chain by *O*-glycosyl residues. The carbohydrate and amino acid analyses of some of these samples used in the present study are given in Table 1. Sialic acid and sulphate contents of these samples are shown in Tables 2 and 4. Some initial characterization of these glycopeptides has already been reported by Bertillini *et al.* (1971a,b) and Kent *et al.* (1978), but further detailed structural analysis is required.

K_m and *V_{max}* values of neuraminidase reaction

The *C. perfringens* neuraminidase used in the

and sulphated glycopeptides were eluted as single components (Fig. 1), ranging between 1.0×10^7 to 1.7×10^7 in mol.wt., and they retained their identity when subjected to rechromatography under similar

Table 2. Extent of hydrolysis of various substrates by *C. perfringens* neuraminidase and their total *N*-acetylneuraminic acid contents

Total AcNeu contents were estimated after hydrolysis for 1 and 24 h of the samples in 0.1 M-H₂SO₄. The reaction was carried out in 0.05 M-sodium citrate buffer, pH 5.0, with a fixed concentration (1.0 µg) of the enzyme for 1 h at 37°C. The rest of the details are given in the text. Each estimation was carried out in duplicate and the results given below are averages of three experiments. All the sulphated glycopeptides did not act as substrates for neuraminidase.

Substrate	Total bound AcNeu in the substrate (µg of AcNeu/mg dry wt. of sample)	Total bound AcNeu in the reaction mixture (µmol)	AcNeu released by neuraminidase	
			(µmol/h)	(% release)
<i>N</i> -Acetylneuraminyl-lactose	408.6	1.75	0.52	30
Bovine submaxillary-gland mucin	47.8	1.74	0.47	27
Sialoglycopeptides				
GLP-400	38.4	1.06	0.23	22
GV.023	122.8	1.60	0.49	31
GB.045	42.6	1.02	0.28	28
Sulphated glycopeptides				
GLPS-283	2.3			
GLPS-341	2.6			
GV.030	7.9			
GB.055	5.3			
GO.075	18.5			
VO.055	17.8			

present study showed optimal activity at pH 5.0 in 0.05 M-sodium acetate/acetic acid buffer with K_m and V_{max} values being $(2.4 \pm 0.004) \times 10^{-3}$ M (mean $\pm$ S.D.) and 0.21 ± 0.015 µmol of *N*-acetylneuraminic acid released/h per mg of protein (mean $\pm$ S.D.) respectively for the hydrolysis of *N*-acetylneuraminyl-lactose as a substrate (Fig. 2). Sialoglycopeptides supplied by Crinos Laboratories acted as substrates for this enzyme and the optimal pH of their hydrolysis reaction being pH 5.0 was similar to that observed for *N*-acetylneuraminyl-lactose and bovine submaxillary-gland mucin when used as substrates. The extent of hydrolysis of these compounds by neuraminidase and their total *N*-acetylneuraminic acid contents determined after hydrolysis for 1 h and 24 h in 0.1 M-H₂SO₄ at 80°C are shown in Table 2.

Inhibition of neuraminidase-catalysed release of sialic acid from various substrates by sulphated glycopeptides

Chemically sulphated glycopeptides were tested for their potential role as an activator or inhibitor in the neuraminidase-catalysed reaction. The ratios of observed rates of *N*-acetylneuraminic acid released from *N*-acetylneuraminyl-lactose and bovine submaxillary-gland mucin in the presence (v) and absence (v_0) of sulphated glycopeptides (GLPS-341 and GLPS-283) are plotted against their concentrations in Fig. 3. Concentrations of sulphated glycopeptides as low as 0.3 µg/ml (in the final reaction mixture), when added 1 min before the addition of the enzyme,

Fig. 3. Effect of sulphated glycopeptides on the hydrolysis of *N*-acetylneuraminyl-lactose and bovine submaxillary-gland mucin by neuraminidase

C. perfringens neuraminidase was incubated with sulphated glycopeptides GLPS-341 (circles) or GLPS-283 (triangles), for 1 min at 37°C before the addition of *N*-acetylneuraminyl-lactose (open symbols) and bovine submaxillary-gland mucin (closed symbols). The enzyme activity was then measured as described in the text. The ratios of *N*-acetylneuraminic acid released in the presence (v) and absence (v_0) of the inhibitor are plotted against its concentration. The values are averages of three experiments.

Table 3. Effect of sulphated glycopeptides on *C. perfringens* neuraminidase inhibition with respect to various substrates. Portions of *C. perfringens* neuraminidase were incubated with various sulphated glycopeptides (0.3 µg/ml of reaction mixture) for 1 min before addition of substrate solutions (1 mmol with respect to *N*-acetylneuraminyl content). The reaction was carried out for 1 h at 37°C. Control enzyme samples in the absence of sulphated glycopeptides were treated in a similar way. The results are expressed as percentage activity inhibited in the presence of the inhibitors. The values given are averages of three experiments and each estimation was in duplicate.

Sulphated glycopeptides	Inhibition of neuraminidase activity measured using different substrates (%)				
	<i>N</i> -Acetylneuraminyl-lactose	Bovine submaxillary-gland mucin	Sialoglycopeptides		
			GLP-400	GV.023	GB.045
GLPS-283	43.5	78.0	76.5	82.0	75.0
GLPS-341	40.0	86.5	80.0	78.5	80.0
GV.030*	37.5	71.5	81.5	76.5	76.0
GB.055†	38.0	76.0	77.5	73.0	80.0
GO.075	37.0	74.0	76.0	81.0	85.0
VO.055	40.0	78.0	80.0	79.5	80.0

* Chemically sulphated derivative of sialoglycopeptide GV.023.

† Chemically sulphated derivative of sialoglycopeptide GB.045.

Fig. 4. Effect of pH on the inhibition of neuraminidase by sulphated glycopeptide

C. perfringens neuraminidase samples were treated with sulphated glycopeptide (GLPS-341, 0.3 µg/ml of reaction mixture) for 1 min at pH values between 3.6 and 6.5 (broken lines). The buffers used were 0.05 M-sodium acetate/acetic acid for pH values between 3.5 and 5.6 and 0.05 M-Na₂HPO₄/NaH₂PO₄ for pH values between 5.8 and 6.5 (Gomori, 1955). The enzyme activity was measured at the same pH values by using *N*-acetylneuraminyl-lactose (○) and bovine submaxillary-gland mucin (●). Controls were run under similar pH conditions in the absence of glycopeptide (solid lines). The results are expressed as percentages of the optimal activity of the control samples. The values plotted are averages of three experiments.

sulphated glycopeptides as high as 300 µg/ml in the reaction mixture did not further increase the degree of enzyme inhibition with respect to various substrates tested.

The inhibition of *C. perfringens* neuraminidase by sulphated glycopeptide tested with two substrates showed a pH-dependent behaviour. The enzyme inhibition at pH values lower than the optimal pH 5.0 was of higher magnitude compared with the inhibition at pH values higher than pH 5.0 (Fig. 4). The sulphated glycopeptides, because of their anionic character, would be more highly charged at pH values lower than 5.0. The increased inhibition would be due to increased interactions between the anionic inhibitor and the cationic groups on the enzyme molecule. The possibility that the enzyme molecule may also be ionized could not be ruled out. Similar results on the inhibition of leucocyte neuraminidase by chondroitin sulphate and heparin had been reported previously (Avila & Convit, 1976).

The relative activity of mixtures of neuraminidase and sulphated glycopeptides in the presence of *N*-acetylneuraminyl-lactose or of bovine submaxillary-gland mucin as substrates indicated (Fig. 2) that the enzyme inhibition in the presence of the sulphated glycopeptides could be a function of the molecular weight and shape of the substrates. To elucidate the mechanism of inhibition, enzyme reactions were carried out where concentrations of both substrates and sulphated glycopeptide were varied. The results obtained (Fig. 5) in the presence of a fixed concentration of inhibitor with various concentrations of the substrates in the reaction mixture ruled out enzyme inhibition (v/v_0) of a competitive type, as the degree of inhibition is independent of substrate concentration. On the other hand, the conventional types of non-competitive

caused about 40 and 86% inhibition of the hydrolysis of *N*-acetylneuraminyl-lactose and bovine submaxillary-gland mucin respectively. A summary of the results with various sulphated glycopeptide samples as inhibitors and other sialoglycopeptides as substrates is given in Table 3. Concentrations of the

Fig. 5. Effect of substrate concentration on neuraminidase inhibition by sulphated glycopeptides

Samples of *C. perfringens* neuraminidase were treated with sulphated glycopeptides GLPS-341 (○ and ●), GLPS-283 (□ and ■), GB.055 (△ and ▲) and GO.075 (▽ and ▼) for 1 min at a concentration of 0.3 µg of inhibitor/ml of reaction mixture. The enzyme activity was measured by using 1–5 mM-*N*-acetylneuraminyl-lactose (open symbols) and 1–5 mg of bovine submaxillary-gland mucin/ml of reaction mixture (filled symbols) at pH 5.0. The ratios of *N*-acetylneuraminic acid released in the presence (v) and absence (v_0) of inhibitors are plotted. The values are means of three experiments.

inhibitions were also contradicted since the plots (Fig. 6) where increasing amounts of an inhibitor in the presence of three different concentrations of the substrates were used are not linear.

Since the behaviour of the neuraminidase/sulphated glycopeptide system did not fall clearly into any of the classical types of inhibition and the degree of inhibition depended on the shape and size of the substrate, it was reasonable to assume that the inhibition was due to steric hindrance as a consequence of non-specific interactions between the enzyme protein and polyanionic sulphated glycopeptides affecting the accessibility of the substrate

Fig. 6. Effect of sulphated glycopeptide concentration on neuraminidase inhibition in the presence of different concentrations of the substrates

C. perfringens neuraminidase samples were treated with sulphated glycopeptide GLPS-341 for 1 min and enzyme reaction was carried out by using 2.5 mM- (○), 5 mM- (□) and 10 mM-*N*-acetylneuraminyl-lactose (△) or 1 mg (●), 2.5 mg (■) and 5 mg of bovine submaxillary-gland mucin/ml (▲) as substrates at pH 5.0. The ratios of *N*-acetylneuraminic acid released in the absence (v_0) and the presence of inhibitor (v) plotted are means of three experiments.

molecules to the catalytic sites. To test the number of accessible active sites, a combination of enzyme-sulphated glycopeptide complex with a competitive inhibitor of the enzyme was examined. In these experiments, neuraminidase from *V. cholerae* was used, since *N*-acetylneuraminic acid acts as a competitive inhibitor with respect to *N*-acetylneuraminyl-lactose (Mohr, 1960). The K_i value calculated from Dixon's plot of $1/v$ against $[I]$ at different substrate concentrations (Dixon & Webb, 1964b) was $1.4 \times 10^{-2} M$ (Fig. 7). The inhibition of *V. cholerae* neuraminidase by sulphated glycopeptide was similar in nature and magnitude to that observed for *C. perfringens* neuraminidase (see the inset of Fig. 8). Enzyme alone, sulphated glycopeptide alone, and a mixture of enzyme and sulphated glycopeptide were dialysed in Visking cellulose tubing against different quantities of *N*-acetylneuraminic acid. The results (Fig. 8) indicated that 1 mol of *N*-acetylneuraminic acid bound to 1 mol of enzyme regardless of the absence or presence of sulphated glycopeptide (at 0.1 or 0.3 µg/ml), which caused respectively 15 and 40% inhibition when measured with *N*-acetylneuraminyl-lactose as substrate or 35 and 85%

Fig. 7. Dixon plots of competitive inhibition of *V. cholerae* neuraminidase by *N*-acetylneuraminic acid

V. cholerae neuraminidase was incubated in the presence of different concentrations of *N*-acetylneuraminic acid (0–40 mM). The enzyme reaction was carried out by the addition of 1 mM- (solid lines) and 0.5 mM-*N*-acetylneuraminyl-lactose (broken lines) as substrate. Velocities were measured as μmol of lactose released/h per mg of enzyme protein. The amount of lactose was determined enzymically by using *Escherichia coli* β -D-galactosidase (EC 3.2.1.23) by the method of Asp & Dahlqvist (1972). The values are averages of three experiments.

Fig. 8. Effect of sulphated glycopeptides on binding of *N*-acetylneuraminic acid, a competitive inhibitor, with *V. cholerae* neuraminidase

V. cholerae neuraminidase (1 mmol) samples in the absence (○) and presence of sulphated glycopeptide GLPS-341 at 0.1 $\mu\text{g}/\text{ml}$ of reaction mixture (△) and 0.3 $\mu\text{g}/\text{ml}$ of reaction mixture (×) were dialysed against 1–5 mmol of *N*-acetylneuraminic acid at room temperature for 24 h. Enzyme samples were then

inhibited respectively with bovine submaxillary-gland mucin as substrate. Hence *N*-acetylneuraminyl-lactose (mol.wt. 633) appears to have had access to the enzyme catalytic site, whereas *N*-acetylneuraminyl-lactose or bovine submaxillary-gland mucin were less accessible. The results, though supporting enzyme inhibition as being due to steric hindrance, cannot conclusively rule out that these inhibitors affected the differential catalysis of the two substrates through formation of the non-productive inhibitor–enzyme–substrate complex. Presence of an inactive enzyme fraction, inactivated by repeated freezing and thawing, with the active enzyme, decreased the inhibitory action of sulphated glycopeptide on neuraminidase activity (Fig. 9). It is noteworthy that the restoration of the enzyme ac-

Fig. 9. Reactivation of sulphated glycopeptide-inhibited neuraminidase activity by pretreatment with inactive enzyme

C. perfringens neuraminidase samples (2 $\mu\text{g}/\text{ml}$ of reaction mixture) were mixed with the inactive enzyme before (solid lines) or after the addition of sulphated glycopeptide GLPS-341 (0.3 $\mu\text{g}/\text{ml}$ of reaction mixture; broken lines). Enzyme activity was determined by using *N*-acetylneuraminyl-lactose (○) and bovine submaxillary-gland mucin (●) as substrates. Results are expressed as percentage reactivation of the enzyme activity. The values are averages of three experiments. Inactive enzyme was prepared by repeated freezing and thawing of active neuraminidase.

precipitated with 10% (w/v) trichloroacetic acid and the precipitates were hydrolysed in 0.1 M- H_2SO_4 for 24 h at 80°C. *N*-Acetylneuraminic acid was determined as described in the text. The inset shows the inhibition of *V. cholerae* neuraminidase by sulphated glycoprotein GLPS-341. The rest of the detail is as described in the legend to Fig. 3.

tivity was achieved only when the inactive enzyme was added before the addition of the inhibitor and without altering the final volume (0.2ml) of the

Fig. 10. Effect of dilution of enzyme/sulphated glycopeptide mixture on reactivation of the neuraminidase activity. *C. perfringens* neuraminidase samples (20 µg/ml of reaction mixture) were diluted immediately after the addition of sulphated glycopeptide GLPS-341 (2 µg/ml of reaction mixture). The enzyme activity was measured by using *N*-acetylneuraminyl-lactose (○) and bovine submaxillary-gland mucin (●). The results are expressed as percentages of the control enzyme activity. The values are averages of three experiments.

enzyme/inhibitor mixture. The addition of inactive enzyme after mixing active enzyme fraction and inhibitor did not restore the enzyme activity by more than 8% (Fig. 9). Many-fold dilution of the concentrated enzyme/inhibitor mixture (20 µg of enzyme + 2 µg of inhibitor/ml of reaction mixture) immediately after their mixing also considerably decreased the enzyme inactivation (Fig. 10). These experiments suggest that the non-specific interaction between the sulphated glycopeptide and the enzyme molecule that caused inactivation of the enzyme could be reversed to a large extent. The restoration of the enzyme activity under the above experimental conditions was dependent on the type of the substrates used.

In an immunochemical investigation it has been reported that neuraminidase inhibition by anti-neuraminidase antibody is due to steric hindrance, depending on the shape and size of the substrate molecule. Enzyme inhibition by antibody was complete against substrate of mol.wt. 10^4 , and hardly measurable against *N*-acetylneuraminyl-lactose (Fazekas de St. Groth, 1963). Similar observations were made (Palese *et al.*, 1973) where anti-neuraminidase antibody and concanavalin A, which inhibited enzyme activity with fetuin as a substrate, had no inhibitory effect on the hydrolysis of *N*-acetyl-2-(3-methoxyphenyl)- α -neuraminic acid. Neuraminidase inhibition (40%), when measured with *N*-acetylneuraminyl-lactose, a low molecular weight substrate, in the presence of sulphated glycopeptides, would indicate that the intermolecular interactions between these polyanionic substances and the cationic

Table 4. Effect of partial removal of sulphate esters from sulphated glycopeptides on their inhibition of neuraminidase activity with respect to *N*-acetylneuraminyl-lactose and bovine submaxillary-gland mucin as substrates

Sulphated glycopeptides (1 mg) were incubated with *Helix pomatia* arylsulphatase (0.5 mg of protein; 2.5 units) at 37°C for 18 h in 0.1 M-sodium acetate buffer, pH 6.2. Samples were then dialysed for 24 h against 0.05 M-sodium acetate buffer, pH 5.0. Similar samples of sulphated glycopeptides were prepared where heat-inactivated arylsulphatase was used. Sulphate contents of both sets of samples were determined. Neuraminidase preparations were incubated with these samples for 1 min at 37°C before addition of the substrate. The reaction was carried out for 1 h and enzyme activities were measured as described in the text. The results are expressed as percentage activity inhibited in the presence of the inhibitors. Each estimation was carried out in duplicate with the appropriate control and the values given are averages of three experiments. Total sulphate contents are expressed as mg of hydrolysable SO_4^{2-} /100 mg of sample.

Sulphated glycopeptide	Total sulphate content (mg/100 mg of sample)	Sulphate removed by arylsulphatase (%)	Inhibition of neuraminidase (%)			
			<i>N</i> -Acetylneuraminyl-lactose as substrate		Bovine submaxillary-gland mucin as substrate	
			Original sulphated glycopeptide	Sulphated glycopeptide treated with arylsulphatase	Original sulphated glycopeptide	Sulphated glycopeptide treated with arylsulphatase
GLPS-283	9.14	22	45	22	80	48
GLPS-341	7.06	49	42	18	84	36
VO.055	9.71	26	39	23	72	32
VO.030	8.97	27	38	24	76	31
GB.055	7.05	35	39	21	77	39
GO.075	7.84	27	40	23	78	50

groups on the enzyme were least specific in comparison with the neuraminidase interaction with its own antibody or with concanavalin A, which is specific for α -D-glucosyl and sterically related residues (Goldstein *et al.*, 1965).

Further evidence to support the view that the steric inhibition of neuraminidase activity by the sulphated glycopeptides was due to their polyanionic property can be drawn from experiments where prior treatment of these inhibitors with *Helix pomatia* arylsulphatase, hence a partial removal of some (approx. 50%) of the sulphate esters, produced a decrease in the degree of neuraminidase inhibition (Table 4). Partially desulphated glycopeptides did not become substrates for the neuraminidase. Further incubations of partially desulphated samples for another 18 h in the presence of fresh addition of arylsulphatase did not remove any more sulphate esters.

Finally it can be concluded that the inhibition of neuraminidase activity was through steric hindrance owing to formation of a sulphated glycopeptide-enzyme complex as a result of intermolecular ionic interactions.

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