DIFFERENCES IN SPLITTING CAPACITY OF VIRUS AND V. CHOLERAE NEURAMINIDASES ON SIALIC ACID TYPE SUBSTRATES

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The only enzyme known to be an integral part of the virus particle and to be coded by the virus genome is the neuraminidase of myxoviruses. This enzyme is also present in some bacteria and vertebrates. It is also called sialidase (N-acetylneuraminate glycohydrolase, E.C. 3.2.1.18) since it liberates sialic acids bound by alpha-ketosidic linkages to carbohydrates (Gottschalk, 1966).

Our experiments demonstrate that viral and V. cholerae neuraminidases differ in substrate specificity. V. cholerae enzyme can hydrolize both 2-6' and 2-3' linkages of sialic acid to carbohydrates whereas the virus readily splits only 2-3' linkages.

METHODS AND RESULTS

The following substrates were tested: 3'-sialyllactose (N-acetyl-neuraminyl-(2-3')-ß-D-galactopyranosyl-(1-4')-D-glucopyranose (Schneir et al., 1962) 6'sialyllactose (6'-lactaminyllactose), 6'-sialyl-N-acetyllactosamine (6'-lactaminyl-N-acetyllactosamine), pentasaccharide b (6'-sialyl-lacto-N-tetraose), pentasaccharide c (6'-sialyl-lacto-N-neotetraose) (Kuhn and Gauhe, 1965).

V. cholerae enzyme was a gift of Behringwerke AG/Marburg. Virus neuraminidases from influenza A2, fowl plague virus and Newcastle disease virus were used as whole virus and as soluble enzymes prepared in a purified form as described previously (Drzeniek et al., 1966). A total amount of 45 ± 5 ug sialic acid as determined by the orcinol method was employed in each test, unless otherwise stated. The virus neuraminidase reaction was performed at pH 7.0 using 0.1 m phosphate buffer, while the V. cholerae neuraminidase was tested at pH 5.5 in 0.05 m acetate buffer containing 0.9% NaCl and 0.1% $CaCl_2 \cdot 2H_2O$. The enzymatic activities used were such that under standard conditions at 37°C 10 ug of sialic acid were liberated from 3'-sialyllactose in 15 minutes which corresponds to 2.15 10^{-3} enzyme units. When tests were kept longer than 2 hours merthiolate was added (0.01%) to avoid a bacterial destruction of the liberated sialic acid. The procedure was described in detail (Drzeniek et al., 1966).

Table 1 lists the amount of sialic acid released in 15 minutes from different substrates. This test procedure corresponds to the initial velocity of the reaction. 3'-sialyllactose was taken as the 100% reference for comparison with the 6'-substrates. In comparison to 3'-substrate the initial velocity of both V. cholerae and viral neuraminidases is sharply reduced using the 6'-substrates.

The differences in enzymatic activity between V. cholerae and virus neuraminidases become obvious when free sialic acid is measured after 24 hours of incubation (Table 2). V. cholerae neuraminidase splits off between

Table 1

Initial reaction velocity of sialic acid release

Substrate	Source of Neuraminidase		
	V. cholerae	Influenza A2	
		Virus	Enzyme
3'-Sialyllactose	100	100	100
6'-Sialyllactose	12[a)]	2	0[*)]
6'-Sialyl-N-acetyllactosamine	24	7	0
Pentasaccharide b[1)]	5	1	2
Pentasaccharide c[2)]	13	0	0

a) All data were expressed as per cent of sialic acid released in 15 minutes by different neuraminidases, considering the hydrolysis of sialic acid from 3'-sialyllactose as a 100 percent.

*) 0 = not detectable

1) 6'-Sialyl-lacto-N-tetraose (2-6' linkage between sialic acid and N-acetylglucosamine of the tetraose)

2) 6'-Sialyl-lacto-N-neotetraose (2-6' linkage between sialic acid and the terminal galactose of the tetraose).

80-100% of sialic acid from all substrates tested. The amount of sialic acid liberated by influenza A2 enzyme, however, is either not detectable or never higher than 7%. This is also true for fowl plague and Newcastle disease virus neuraminidases, which suggests that the highly reduced capacity or inability to attack 2-6' linkages of sialic acid containing substrates is a characteristic feature of Myxovirus neuraminidases.

Table 2

Sialic acid released after prolonged treatment

Substrate	Source of Neuraminidase		
	V. cholerae	Influenza A2	
		Virus	Enzyme
3'-Sialyllactose	100	100	100
6'-Sialyllactose	85[a)]	2	0
6'-Sialyl-N-acetyllactosamine	99	4	3
Pentasaccharide b	81	2	7
Pentasaccharide c	87	1	5

a) All data were expressed as per cent of sialic acid released in 24 hours by different neuraminidases considering the hydrolysis of sialic acid from 3'-sialyllactose as a 100 per cent.

It was previously shown that the pH-optimum of neuraminidases is dependent on the substrate used (Mohr and Schramm, 1960), therefore the tests were done at different pH-values. Nevertheless, higher amounts of sialic acid cannot be split off from 6'-derivatives if the pH-values are lowered, Table 3. Incubation for longer periods at pH $\leq$ 5 is not possible due to the lability of the enzyme.

In order to exclude that the substrate specificity recorded only exists with an arbitrarily chosen enzyme/substrate ratio, higher concentrations of influenza A2 virus neuraminidase were used in some tests. Figure 1 shows that higher enzymatic activities are not more efficient in splitting 6'-compounds. In contrast to the hydrolysis of 3'-sub-

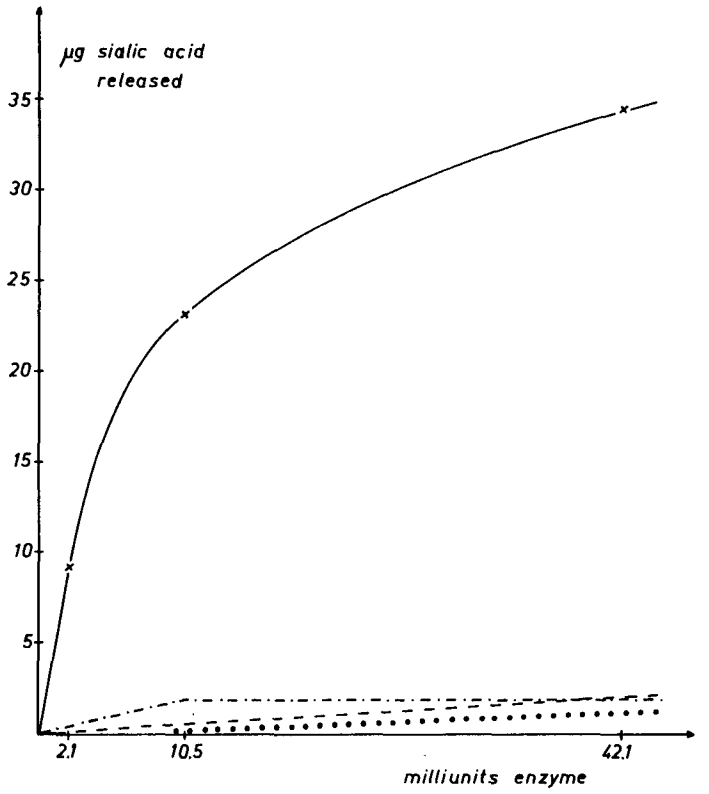

Fig. 1. Release of sialic acid from different substrates using influenza A2 virus neuraminidase after 15' incubation at pH 7.0, expressed as µg sialic acid/0.5 ml test volume by 2.1, 10.5, and 42.1 x 10^{-3} enzyme units / 0.5 ml, respectively. Details of the test procedure were described (Drzeniek et al., 1966).
x——x 3'-sialyllactose (3'-SL); .... 6'-sialyllactose (6'-SL)
------ 6'sialyl-N-acetyllactosamine (6'-SALA), pentasaccharide c (PSc); -.-.-.-.-. pentasaccharide b (PSb).

strates no more than 7% of sialic acid was liberated from 6'-substrates. The problem arose whether the differences reside in the binding or hydrolytic capacities of the enzyme. Inhibitory experiments demonstrated that after addition of 6'-compounds to the 3'-sialyllactose used as substrate, a competitive inhibition of the viral enzymes occurred. This indicates that the 6'-compounds are bound to the viral enzyme but they are not cleaved efficiently.

Table 3

Sialic acid released by influenza A2 neuraminidase from various substrates at different pH

pH	3'-Sialyllactose	6'-Sialyllactose	6'-Sialyl-N-acetyl-lactosamine	Pentasaccharide c
7.0	100	2[a)]	7	1
6.0	100	0	5	2
5.0	91.5	0	5	3
4.0	76	0	5	3

a) All data were expressed as per cent of sialic acid released in 15 minutes by influenza A2 virus neuraminidase at the indicated pH value considering the hydrolysis of sialic acid at pH 7.0 from 3'-sialyllactose as a 100 per cent.

DISCUSSION

The results of our experiments show that in contrast to V. cholerae Myxovirus neuraminidases can barely attack compounds which carry the sialic acid in an alpha-ketosidic linkage to the 6'-C-atom of the carbohydrate. It is not certain whether the slight activity measured is due to the presence of a hydrolyzable contamination or to a residual activity of the viral enzyme towards 6'-compounds.

Our inhibitory experiments demonstrate that the rate limiting factor of the enzymatic process is not the enzyme-substrate binding but the hydrolysis of the substrate. These findings indicate that all sialic acid-containing substrates could potentially function as virus receptors. Differences can be expected, however, with respect to the ease at which the virus can elute from different receptors by splitting off sialic acid. These differences might have some significance for virus penetration, since infectious particles are attached for a longer time to 6'-receptors and thus have a better chance to enter the cell. On the other hand a virus particle might readily be released from cells if it attaches to 3'-receptors. Further experiments with biologically interesting substances are planned in vitro and in vivo to clarify these questions.

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