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Specificity studies on the oligosaccharide neuraminidase of human fibroblasts

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Competition and thermal inactivation experiments with different potential natural substrates indicated that in homogenates of human fibroblasts one single enzyme is acting on both (α2-3) and (α2-6) sialosyl linkages of oligosaccharides and glycoproteins, but not of the ganglioside G_{M3}. *N*-Acetylneuraminic and 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acids are competitive inhibitors, whereas chondroitin 4-sulphate and the drug Suramin are potent inhibitors of undefined type.

Cultured human fibroblasts are thought to contain at least two genetically distinct neuraminidases: an oligosaccharide-specific enzyme, deficient in the lysosomal storage disease sialidosis, and a ganglioside-specific hydrolase which is lacking in another such disorder, i.e. mucolipidosis IV (Wenger *et al.*, 1978; Cantz & Messer, 1979; Bach *et al.*, 1979). As these enzymes are membrane-bound and rather labile, attempts at purification and characterization proved difficult. Although the study of the sialidoses indicated that the deficient oligosaccharide neuraminidase would be specific for both (α2-3) and (α2-6) sialosyl linkages (O'Brien, 1977; Frisch & Neufeld, 1979; Cantz & Messer, 1979), it was not clear whether the deficiency involved a single enzyme, or perhaps a system of oligomeric isoenzymes analogous to the situation in Sandhoff and Tay-Sachs diseases (O'Brien, 1983).

Using natural substrates, some of which were ^{3}H-labelled, we have now performed competition and thermal inactivation experiments to study the specificity of the oligosaccharide neuraminidase in fibroblast homogenates and, by inference, the metabolic defect in sialidosis. In addition, the sensitivity of the enzyme towards the action of various inhibitors was tested. The results indicate that one single enzyme is acting on both types of sialosyl linkages of oligosaccharides and glycoproteins.

Abbreviations used: neuraminidase, acylneuraminyl hydrolase, EC 3.2.1.18; II^3-sialyl-lactose, NeuAc(α2-3)-Gal(β1-4)Glc; II^6-sialyl-lactose, NeuAc(α2-6)Gal(β1-4)-Glc; ganglioside G_{M3}, NeuAc(α2-3)Gal(β1-4)Glc(β1-1)-Cer; Cer, ceramide; $C_{12}E_{10}$, polyoxyethylene(10)-dodecylether; Suramin, 8,8′-{carbonylbis[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)-carbonylimino]}bis-1,3,5-naphthalenetrisulphonic acid hexasodium salt. Ganglioside nomenclature is according to Svennerholm (1963).

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Materials and methods

Ganglioside G_{M3} was a gift from Dr. R. Veh, Ruhr-Universität, Bochum, Germany. Sodium chondroitin 4-sulphate and its sulphated tetrasaccharide, (GlcA-GalNAc-4-sulphate)$_2$, were prepared as described (Gorham & Cantz, 1978). 2-Deoxy-2,3-dehydro-*N*-acetylneuraminic acid and Suramin were gifts of Boehringer Mannheim and Bayer Leverkusen, respectively.

Fetuin, type IV, and bovine submaxillary mucin, type I, were from Sigma. Their sialic acid content was determined by incubating 0.25mg of glycoprotein in 200μl of 50mM-sodium acetate buffer, pH5.5, containing 10mM-$CaCl_2$, 15mM-NaCl, and 15m-units of neuraminidase (*Vibrio cholerae*, Serva, Heidelberg) at 37°C for 18h, and the liberated sialic acid determined (Skoza & Mohos, 1976). For fetuin, the sialic acid content (as Neu5Ac) was 11mol/mol of glycoprotein, assuming a molecular mass of 48400 (Graham, 1972); for bovine submaxillary mucin, we found 38mol of Neu5Ac/mol, based on a molecular mass of 375000 (Gottschalk *et al.*, 1972). In general, fetuin contains about equal amounts of (α2-3)- and (α2-6)-linked sialic acid (Spiro & Bhoyroo, 1974; Baenziger & Fiete, 1979), whereas the sialosyl linkages of submaxillary mucins are nearly exclusively of the (α2-6) type (Pigman, 1977).

All other reagents were of the highest available purity.

Human skin fibroblasts were maintained in

culture as described (Cantz *et al.*, 1972). Homogenates were prepared and their protein concentrations determined as reported (Cantz & Messer, 1979).

The II^3- and II^6-isomers of sialyl-lactose used as substrates were prepared from cow colostrum and characterized as described (Schneir & Rafelson, 1966). Batches of both compounds were labelled by reduction with NaB^3H_4 (NEN, Dreieich, Germany; 7.902Ci/mol) (Frisch & Neufeld, 1979), yielding specific radioactivities of 42.9 and 23.1 Ci/mol for the II^3- and II^6-isomers, respectively.

The activity of the oligosaccharide neuraminidase was determined in incubations containing 1.16nmol of ^{3}H-labelled II^3-sialyl-lactitol (80000c.p.m.), variable amounts of unlabelled II^3-sialyl-lactose to vary substrate concentration, 15μmol of potassium acetate buffer, pH4.2, 1.4mg of $C_{12}E_{10}$ (Sigma) and 25μg of fibroblast homogenate protein in a total volume of 140μl. After 120min incubation at 25°C, the liberated [^{3}H]lactitol was separated from unreacted substrate by chromatography on mini-columns (0.5cm × 5cm) of Bio-Rex-5, acetate form (Bio-Rad), and the water-elutable radioactivity was determined.

Results and discussion

By using the labelled trisaccharide II^3-sialyl-[^{3}H]lactitol as the reporter substrate, the following compounds were found to act as competitive inhibitors for the neuraminidase activity of the fibroblast homogenates (Fig. 1): II^6-sialyl-lactose, $K_i = 1.06$mM; fetuin, $K_i = 0.23$mM; bovine submaxillary mucin, $K_i = 0.0071$mM. By comparison, the inhibitory effect of the ganglioside G_{M3} was rather weak ($K_i = 6.94$mM). The high affinity of the 'inhibitors' fetuin and bovine submaxillary mucin towards the active site of the neuraminidase may be taken as an indication that they are substrates for one and the same enzyme acting on II^3-sialyl-lactose. For II^6-sialyl-lactose, the K_i value indicates a somewhat lower affinity; this value, however, is close to that of the K_m for II^3-sialyl-lactose (0.66mM), thus suggesting the II^6-isomer to be cleaved by the same active site. On the other hand, the low affinity of the ganglioside under conditions which are suitable for its cleavage (Cantz & Messer, 1979) speaks against it being a substrate.

Additional information on the substrate specificity was expected from thermal inactivation studies of the neuraminidase activity, assuming that different rates of inactivation would result if different enzymes would be acting on different substrates. Fig. 2(*a*) shows that the time-course of thermal inactivation at 50°C of the activity against both isomers of sialyl-lactitol was identical (as was observed when the inactivation was performed at

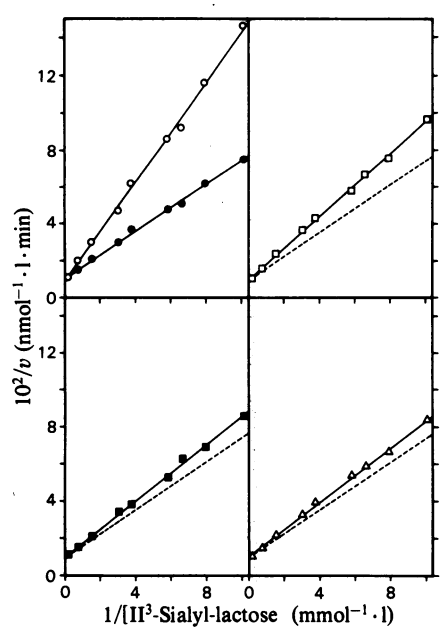

Fig. 1. *Competition of potential substrates with the reporter substrate II^3-sialyl-lactose*

The 'inhibitors' and their concentrations were the following: ○, II^6-sialyl-lactose, 1.58mM; ■, fetuin, 59μM; □, bovine submaxillary mucin, 2.88μM; △, ganglioside G_{M3}, 1.12mM. The symbol ● and the broken line denote reaction in the absence of inhibitor.

37°C; results not shown). The same was found when fetuin was compared with II^3-sialyl-lactitol (Fig. 2*b*).

The oligosaccharide neuraminidase was also studied with regard to its sensitivity towards inhibitors, which are not themselves substrates. As shown in Fig. 3, the glycosaminoglycan chondroitin 4-sulphate produced a strong inhibition that was however limited to a fixed, substrate concentration-dependent value. The reaction was inhibited in the same fashion and to a similar extent when II^6-sialyl-lactose was used as the substrate (results not shown). It is unlikely that this incomplete inhibition is due to the presence of isoenzymes exhibiting different inhibitor sensitivities, because of the strong correlation of the residual activity of the maximally inhibited enzyme with the substrate concentration. Rather, the limitation of the inhibition to a finite value may be attributed to some type of partial inhibition. A disulphated tetrasaccharide prepared from chondroitin 4-sulphate, on the other hand, was only weakly inhibitory, pointing to the importance of the polysaccharide chain length in the interaction with the enzyme. Another polyanionic compound, the trypanocidal drug Suramin, a known inhibitor of a variety of

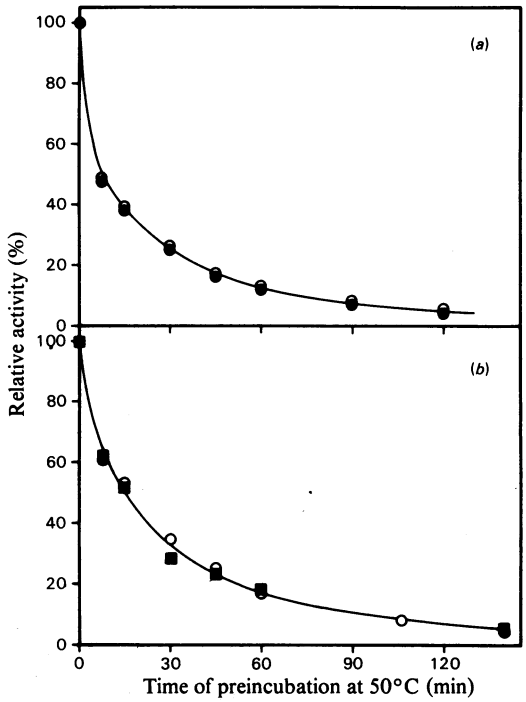

Fig. 2. *Thermal inactivation of neuraminidase activity*
Homogenates were preincubated for various lengths of time at 50°C. Neuraminidase activity towards sialyl-lactose was determined at substrate concentrations of 8.26 μM and 7.73 μM for the II[3] (○) and II[6] (●) isomers, respectively. With fetuin (■) as substrate, 3mg was in 250μl of 0.1M-sodium acetate buffer, pH4.2, containing 2.5mg of $C_{12}E_{10}$ and 300 μg of homogenate protein. After 4h at 25°C, the liberated sialic acid was determined after ion-exchange chromatography on mini columns, as described (Baumkötter & Cantz, 1983). Enzyme activity was calculated after subtraction of homogenate and substrate blanks. In (*a*) the homogenate protein concentration was 2.7mg/ml; in (*b*) it was 3.3mg/ml.

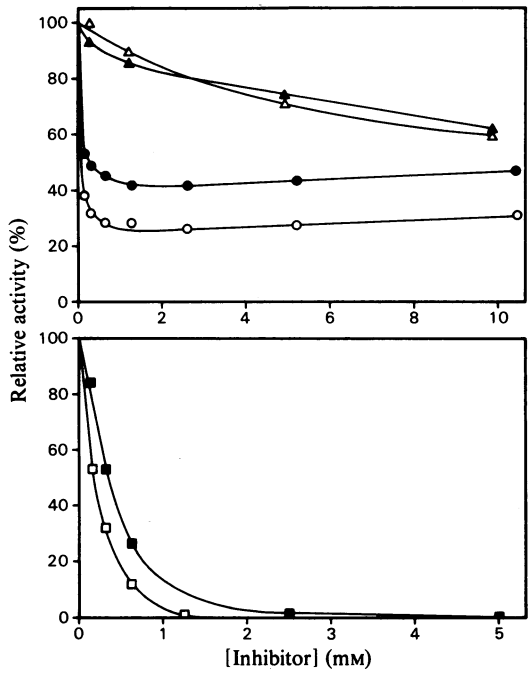

Fig. 3. *Inhibition of oligosaccharide neuraminidase by polyanionic compounds*
Incubations were performed at two substrate concentrations: open symbols, 8.3 μM; filled symbols, 16.6 μM. ○, ●, Chondroitin 4-sulphate; △, ▲, chondroitin 4-sulphate tetrasaccharide; □, ■, Suramin.

lysosomal enzymes (Constantopoulos *et al.*, 1980), was likewise an efficient inhibitor (Fig. 3). The reaction product *N*-acetylneuraminic acid was a fairly strong, and 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, an inhibitor of viral, bacterial and mammalian neuraminidases (Corfield & Schauer, 1982), was a very powerful, competitive inhibitor. The inhibitory potencies of all the compounds studied are summarized in Table 1.

As not only *N*-acetylneuraminic acid, but also 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, have been shown to be present in mammalian tissues (Corfield & Schauer, 1982), it is conceivable that they are of physiological importance in the regulation of the catabolism of sialoglycoconjugates. Chondroitin 4-sulphate and Suramin were also found to inhibit the ganglioside G_{D1a}-specific neuraminidase of fibroblasts, albeit with different kinetics, whereas 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid was moderately inhibitory (I_{50} value 0.98mM), and *N*-acetylneuraminic acid not at all inhibitory (Baumkötter & Cantz, 1983). The latter compounds may therefore be used in the differentiation of the two neuraminidases.

Taken together, the results suggest that the oligosaccharide neuraminidase activity of human fibroblasts is associated with a single enzyme specific for both (α2-3) and (α2-6) sialosyl linkages of oligosaccharides as well as glycoproteins. They speak against the existence of separate neuraminidases for different types of isomeric linkages, as had been proposed by Strecker & Michalski (1978). On the other hand, the enzyme acting on oligosaccharides seems not active towards the (α2-3) sialosyl linkage of ganglioside G_{M3}. Evidence for distinct properties of oligosaccharide- and ganglioside-specific neuraminidases of cultured fibroblasts was also presented in substrate competition experiments using the artificial substrate 4-methyl-

Table 1. *Inhibitors of oligosaccharide neuraminidase*

I_{50} denotes inhibitor concentration causing 50% inhibition under the conditions of assay; K_i is calculated from regression analysis of Dixon plots.

Compound	Type of inhibition	I_{50} (mM)	K_i (mM)
N-Acetylneuraminic acid	Competitive	1.1	1.2
2-Deoxy-2,3-dehydro-*N*-acetylneuraminic acid	Competitive	0.004	0.007
Chondroitin 4-sulphate	?	0.11*	-
Suramin	?	0.16	-

* Based on concentration of disaccharide units.

umbelliferyl *N*-acetylneuraminic acid, sialyl-lactose, and ganglioside G_{D1a} (Caimi *et al.*, 1981), and in subcellular fractionation studies (Zeigler & Bach, 1981).

Our conclusions are in accordance with the above-cited observations in sialidosis (mucolipidosis I) and indicate that the deficiency of a single neuraminidase causes a block in the lysosomal catabolism of sialyloligosaccharides and -glycoproteins, whereas the degradation of gangliosides remains unimpaired, due to the presence of a genetically different, glycolipid-specific neuraminidase.

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