Effect of Temperature on the Activity and Kinetics of Brain Membrane Bound Neuraminidase

A peculiar feature of brain membrane bound neuraminidase is the ability to act at unusually high temperatures[1]. Indeed the maximum rate of the enzymatic hydrolysis of gangliosides – the putative physiological substrates of the bound neuraminidase – is reached at 70 °C[2]. Thus we considered it worth investigating in detail the behaviour of brain membrane bound neuraminidase at 70 °C. The substrate used was disialoganglioside GD1 a (NANα2–3 Galβ1–3 GalNAcβ1–4 (NANα2–3)Galβ1–4 Glcβ1–1 Cer; name according to SVENNERHOLM[3]).

Materials and methods. The membrane-bound neuraminidase was prepared from the 0–105,000 × *g* (1 h) sediment of calf brain homogenate according to PRETI et al.[2]. The preparation was rid of the soluble and lysosomal neuraminidases and was depleted of endogenous substrates as described by TETTAMANTI et al.[4]. Ganglioside GD1 a was prepared from beef brain by the method of TETTAMANTI and ZAMBOTTI[5]. The assay of brain membrane bound neuraminidase followed the general indications of PRETI et al.[2]. Shortly, the incubation mixtures, containing, in a final volume of 0.65 ml, 0.5–1.0 mg enzyme (as protein), 0.15 *M* sodium acetate buffer and adequate amounts of substrate, were set up at melting ice temperature, then immersed into the incubating shaker (adjusted to the desired temperature). The incubation was stopped by immersing the tubes into an acetone dry ice bath. Then the liberated N-acetylneuraminic acid was determined and the corresponding enzyme activity calculated as described by TETTAMANTI et al.[4]. The control incubation mixtures (blanks) were performed using boiled (15 min) enzyme. The temperature inside the incubation mixtures was measured by an Ysi telethermometer (model 43 TC) provided with a proper thermistor probe. One unit of neuraminidase is the amount of enzyme which liberates 1 nmole N-acetylneuraminic acid/min. The protein content was determined by the method of LOWRY et al.[6] with bovine serum albumin as the standard.

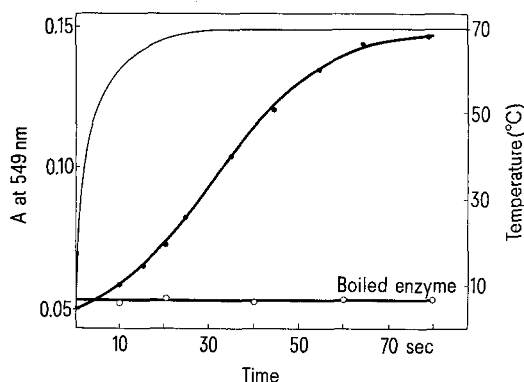

Fig. 1. Time course of the activity of calf brain membrane bound neuraminidase at 70 °C. The assay mixtures contained 870 µg of protein and 0.2 m*M* disialoganglioside GD1a. They were set up in an ice bath, then immersed in the incubating shaker, adjusted to 70 °C and allowed to incubate. The thin line indicates the temperature measured inside the text tubes. The data shown are the average of 4 experiments.

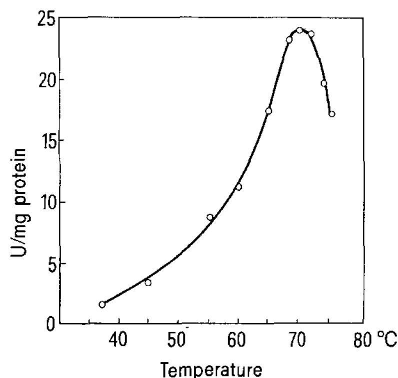

Fig. 2. Change of the hydrolysis rate of disialoganglioside GD1a by calf brain membrane bound neuraminidase with increasing temperature. The assay mixtures, containing 780 µg protein, were incubated at pH optimum for each temperature and for a period of time within the linear course of the enzyme catalyzed reaction. The data shown are the average of 6 experiments.

Results and discussion. The graphs reported in Figure show that the measuring method is really functioning also at the high temperatures (70 °C). The enzymatic liberation of N-acetylneuraminic acid was recorded immediately after the immersion of the incubation mixture in the incubation bath, adjusted to 70 °C. The hydrolysis rate became maximal after 25–30 sec from the immersion, that is after the incubation mixture reached the temperature of the bath. The maximum rate was then maintained for approximately 10 sec (from 25 to 35 sec after the immersion). Afterwards the reaction rate tended, as expected, to diminish. The change of hydrolysis rate of ganglioside GD1 a by membrane-bound neuraminidase with increasing temperature is graphically shown in Figure 2. The specific activity shifted from 1.5 U/mg protein at 37 °C to the maximum of 24 U at 70 °C; then it slowed down probably because of the onset of thermal degradation phenomena. The degree of maintenance of the enzyme activity at 70 °C, as shown in Figure 1, was low. It still diminished on lowering the pH of the incubation mixture; the loss of activity after standing at 70 °C for 30 sec was 38% at pH 6.5, 40% at pH 5.0, 43% at pH 4.7, 75% at pH 3.5. Conversely, it was significantly enhanced by the presence of substrate, ganglioside GD1a. As shown by the graphs of Figure 3, the enzyme activity started diminishing significantly later, and was thereafter maintained at a much higher level in the presence than in the absence of substrate.

The protective effect of ganglioside GD1a depended on the concentration, which was optimum in the range 0.1–0.2 m*M*. Also a non-gangliosidic substrate, like sialyllactose, provided a substantial effect of enzyme protection at high temperatures.

The kinetics of the enzyme catalyzed hydrolysis of ganglioside GD1a at the various temperatures always followed the classical hyperbolic shape and showed the

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Changes with increasing temperature of some kinetic parameters of calf brain membrane bound neuraminidase

Parameter	Temperature (°C)			
	37°	45°	55°	70°
Optimum pH	4.0	4.2	4.4	4.7
V_{max} (units/mg protein)	1.5	3.9	8.1	24
K_m ($\times 10^{-5}$ M)	2.1	3.0	5.8	10.4
Substrate concentration at which the inhibitory effect starts (mM)	0.2	0.25	0.35	0.5
Inhibition at 1 mM substrate (%)	65	50	35	15

Substrate used: disialoganglioside GD1a. The data reported are the average of 5 experiments

well-known inhibition[4,7] by excess substrate. The variations of some kinetic data (optimum pH, V_{max}, K_m, inhibition by excess substrate) with increasing temperature are exposed in the Table. From 37 °C to 70 °C the optimum pH shifted from 4.0 to 4.7, the V_{max} from 1.5 units/mg protein to 24 units, the K_m from 2.1×10^{-5} to 10.4×10^{-5}. The inhibition by excess substrate started at 0.2 mM ganglioside GD1a at 37 °C, at 0.5 mM at 70 °C. The inhibition at 1.0 mM ganglioside GD1a was 65% at 37 °C and only 15% at 70 °C.

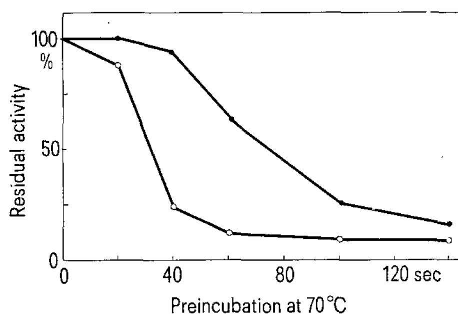

Fig. 3. Effect of the presence of substrate on the stability of calf brain membrane bound neuraminidase at 70 °C. The enzyme preparation was allowed to stand at 70 °C for the indicated time in the presence of 0.15 mM disialoganglioside GD1a (at pH 4.7), then refrigerated in an ice bath and submitted to incubation at 37 °C for 15 min (pH 4.0). The assay mixture contained 880 μg of protein. The data exposed are the average of 4 experiments. -□-□-□-, preincubated in the absence of ganglioside GD1a; -■-■-■-, preininbated in the presence of ganglioside GD1a.

The same behaviour with increasing temperature was shown also with ganglioside GD1b and GT1b as the substrates, and was provided too by the enzyme prepared from the same animal according to LEIBOVITZ and GATT[8]. Also, closely similar findings were observed using the rabbit and human brain enzyme. Thus it can be concluded that the above behaviour is a basic property of brain membrane bound neuraminidase[9].

Riassunto. La velocità di idrolisi del ganglioside GD1a da parte della neuraminidasi di membrana del cervello di vitello è massima a 70 °C. L'aumento della temperatura da 37° a 70 °C provoca: aumento della V_{max} (da 1.5 unità a 24 unità/mg di proteina) e del pH ottimale (da 4.0 a 4.7); aumento del valore di K_m (da 2.1×10^{-5} M a 10.4×10^{-5} M); diminuzione dell'effetto inibitorio da eccesso di substrato.

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Protection of Vaccinia-Infected HeLa Cells by Lipophilic Benzimidazole Derivatives

Although some activities have been reported[1], the picornaviral-inhibiting derivatives of 2-(α-hydroxybenzyl)benzimidazole (HBB) have tended to be classed as inactive, or of low activity, towards DNA viruses[2]. We now find that some of the more lipid-soluble derivatives of HBB can be very effective at inhibiting the onset of cytopathic effect (CPE) in vaccinia-infected monolayers, but this effect depends on the tissue culture system employed. We have reported a similar phenomenon in relation to herpes simplex virus, D-5-chloro-HBB showing activity in ERK and Hep 2 cultures, but none in HEL cultures[1].

Medium [Eagle's minimum essential medium containing foetal bovine serum (10% v/v), $NaHCO_3$ (0.112% w/v), glutamine 2.0 mM, benzyl-penicillin (100 U/ml) and streptomycin (100 μg/ml)] was removed from HeLa monolayers and replaced by fresh medium [with the serum reduced to 2% and the $NaHCO_3$ increased to 0.224%] containing one of a set of neurovaccinia virus dilutions and containing test- compound at the chosen concentration. The virus was included in this medium immediately before its addition to the monolayers. Infected and uninfected control cultures were simulta-

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